

Monograph on Lecithin-Volume 2-Copies of articles cited

#121 12/10/74

MONOGRAPH ON LECITHIN #121 VOL II
COPIES OF ARTICLES CITED

L17

VOLUME 2

GRAS MONOGRAPH SERIES

LECITHIN

**(COPIES OF ARTICLES CITED IN
MONOGRAPH SUMMARY)**

**prepared for
THE FOOD AND DRUG ADMINISTRATION
DEPARTMENT OF HEALTH, EDUCATION
AND WELFARE**

OCTOBER 10, 1974

**This publication was prepared under Contract Number FDA 72-100
with the Public Health Service, Food and Drug Administration,
Department of Health, Education, and Welfare**

**prepared by
Tracor Jitco, Inc.**

Negative Results of Attempts to Prolong the Effect of Phospholipids (PL) and Cholesterol (Chol) on the Formation of Tumours Induced by 3-Methylcholanthrene (MeC) *

R. F. A. ALTMAN, D. J. DA SILVA, and C. R. N. LOPES

Instituto Oswaldo Cruz, Rio de Janeiro, Brazil

(Z. Naturforsch. 25 b, 229—230 [1970]; eingeg. am 18. September 1969)

It was formerly demonstrated that PL retard and Chol accelerates the formation of tumours induced by MeC^{1,2}. This retardation respectively acceleration, however, never surpassed a period of somewhat more than four weeks. The question, then, arose why PL are only capable to retard temporarily and not to prevent, i.e. retard permanently, the formation of tumours. It was presumed that the administration of an excess of PL could favourably influence the time of retardation, namely by impeding the liberation of MeC from its molecular associations with PL through the interaction of Chol occurring everywhere in the organism:



A series of attempts have, therefore, been undertaken in order to increase respectively decrease the PL/Chol-ratio in the organism by the administration of excessive quantities of PL respectively Chol. The obtained results were all negative for neither the retardation by excess of PL, nor the acceleration by excess of Chol of the formation of tumours could be prolonged for more than four weeks.

Method and Results

Eleven groups of twenty Swiss mice each were formed.

The controls (Group I) were injected subcutaneously in one single dose with 0.5 mg MeC in 0.2 ml tri-octanoin (Eastman-Kodak). Group II received 0.5 mg MeC plus 50 mg of "Asolectin" ** in 0.2 ml tri-octanoin. This same injection was applied in the mice of Groups III, IV, V and VI to which, however, additional quantities of PL were given, namely: to Group III, already 35 days before the application of MeC, a 1% Asolectin-emulsion as the drinking water which corresponds with a daily dose of about 1.5 g PL/kg bodyweight; to Group IV: starting 15 days before the MeC-injection, three times weekly 0.1 ml of a 10% emulsion of Asolectin in saline 0.9% subcutaneously, corresponding with a dose of 1 g PL/week/kg bodyweight; to Group V: initiating 15 days before the MeC-dosage, equally three times weekly 0.1 ml of the same Asolectin-emulsion, intraperitoneally; and to Group VI: 0.1 ml of a

2% phosphatidylcholine-emulsion three times weekly in the tail-vein, corresponding with 200 mg PL/week/kg bodyweight.

The animals of Group VII were injected subcutaneously with a single dose of 0.5 mg MeC plus 20 mg Chol dissolved in 0.2 ml tri-octanoin, whereas those of Group VIII received, in addition to that injection and starting already 35 days before the MeC-administration, 4 g Chol dissolved in 80 ml babassu-fat *** per kg of ration which corresponds with a dose of about 1 g Chol/day/kg bodyweight. The additional quantities of Chol for Groups IX and X were administered subcutaneous respectively intraperitoneally three times weekly in doses of 0.1 ml of a 5% Chol-solution in babassu-fat slightly warmed at 40 °C to prevent Chol-crystallisation. This dose corresponds with about 500 mg Chol/week/kg bodyweight. Group XI, finally, received the additional dose of Chol intravenously in the form of an O/W-emulsion, containing 0.8% Chol and stabilized with sodium stearate. This emulsion was introduced three times weekly in a dose of 0.1 ml which corresponds with 75–100 mg Chol/week/kg bodyweight.

The appearance of tumours was controlled twice weekly from the 3rd to the 7th week and, thereafter, three times a week. The results are summarized in the Table 1, in which the three figures a/b/c represent: (a) the total number of living mice, (b) the number of living tumour-bearing mice, and (c) the number of mice died from cancer.

Discussion

It is seen from the figures in the Table 1 that in the twelfth week after MeC-injection, already half of the control mice had acquired cancer, whereas only 15% of Group II, about 36% of Group III, 22% of Group IV, and 28% of Group V beared tumours. In the Chol-groups, on the contrary, these percentages amount to not less than 89 for Group VII, 90 for Group VIII, 95 for Group IX, and 94 for Group X.

Thus, with the exception of the mice which have received either PL or Chol intravenously, it can be positively stated that PL retard the appearance of tumours, whereas Chol showed a pronounced accelerating activity. The same is true for the rate of mortality. Thirty weeks after the MeC-injection, namely, one animal of the control-group, three of Group II, one of Group III and two of Group IV escaped tumour-formation and survived, whereas already 16 weeks after MeC-administration, all mice of the Chol-groups have died from cancer, excepted those of Group VII which, however, were all tumour-bearing and, hence, condemned to death. The mice of Groups IV, V, IX and X

* Supported by a grant of the Brazilian National Research Council (Conselho Nacional de Pesquisas), Rio de Janeiro.

¹ R. F. A. ALTMAN, O. PUGACHOV, I. BALLINI-KERR, and D. J. DA SILVA, Z. Naturforsch. 23 b, 1277 [1968].

² R. F. A. ALTMAN, O. PUGACHOV, I. BALLINI-KERR, and L. L. S. PINTO, Arch. Geschwulstforsch. 31, 133 [1968].

** This granulated soybean phosphatide was gently put at our disposal by Mr. J. EICHBERG, President of American Lecithin Co., Atlanta, USA.

*** Babassu-fat was gently put at our disposal by Dr. MOACYR SILVA, Technical Director of "Carioca Industrial, S.A.", Rio de Janeiro.

Group	Weeks	7 a/b/c	10 a/b/c	12 a/b/c	16 a/b/c	25 a/b/c	30 a/b/c
I (Contr.)		18/4/0	18/7/0	18/9/0	14/10/4	3/2/15	1/0/17
II (PL, once only)		20/2/0	20/2/0	20/3/0	19/10/1	3/0/17	3/0/17
III (PL, oral)		14/0/0	14/4/0	14/5/0	14/10/0	2/1/13	1/0/14
IV (PL, s. c.)		18/1/0	18/2/0	18/4/0	18/11/0	4/2/14	2/0/16
V (PL, i. p.)		18/2/0	18/3/0	18/5/0	14/12/4	1/1/17	0/0/18
VI (PL, i. v.)		9/6/0	8/3/1	8/5/1	4/4/4	0/0/8	—
VII (Chol, once)		19/12/0	19/17/0	18/16/1	8/8/11	0/0/18	—
VIII (Chol, oral)		20/7/0	20/15/0	20/18/0	0/0/20	—	—
IX (Chol, s. c.)		19/7/0	19/15/0	19/18/0	0/0/19	—	—
X (Chol, i. p.)		16/7/0	16/13/0	15/14/1	0/0/16	—	—
XI (Chol, i. v.)		2/0/0	suspended				

Table 1. Appearance of tumours after subcutaneous MeC-administration.

must have suffered from the received subcutaneous and intraperitoneal injections for a large excess of unabsorbed PL-emulsion, respectively oil-solutions of Chol, was constantly found either subcutaneously or in the abdominal cavity.

A high mortality was observed in the intravenously injected mice, probably as a consequence of the imperfect condition of the introduced emulsions due to lack of an adequate stabilizer which, in the meantime, has been found in Pluronic F-68, a non-ionic, water-soluble polyoxyethyleneoxypropylene polymer produced by Wyandotte Chemical Corp., Wyandotte, USA³.

It seems worth while to repeat the introduction of either PL or Chol directly in the bloodstream for the excess of these lipids when administered by other ways has not shown the slightest effect, probably due to metabolic modifications of their molecular structures. However it may be, it must be concluded from the

above that the activity of PL and Chol is only perceptible when these compounds find themselves in direct molecular contact with the carcinogen. This confirms the assumed existence of molecular associations of PL and MeC from which MeC can be released by Chol, due to the higher affinity of the latter to PL (see the equation given in the Introduction). As was already explained before⁴, PL-MeC associations are too large in size for occupying open spaces in the cell-membranes (cf. ALTMAN^{4,5}).

Experiments are now in progress to verify the activity of a large excess of PL and Chol introduced *together* with the carcinogenic hydrocarbon. At the same time the influence of an excess of these lipids administered intravenously in the form of perfect emulsions is studied.

Thanks are due to V. C. DO AMARAL for his invaluable technical assistance.

³ P. E. SCHURR, Cancer Res. 29, 258 [1969].

⁴ R. F. A. ALTMAN, Arch. Geschwulstforsch. 19, 1, 97 [1962].

⁵ R. F. A. ALTMAN, O Hospital [Rio de Janeiro] 73, 1525 [1968].

Committee on Specifications. 1972
Food Chemicals Codex
Committee on Food Protection, National Academy of Sciences,
National Research Council, Washington, D.C.
pp. 382-385; 444-446

Effect of Lecithin on Glucose Metabolism in Normal Dogs

by E. Couturier and V. Conard

In a previous article, we studied the influence of triglycerides and non-esterified plasmic fatty acids on glycemia and insulinemia and the speed of the usage of glucose in normal anesthetized dogs (1).

Elevation in the lipid level is obtained by perfusing a solution of triglycerides either with or without an injection of heparin. While an increase in triglycerides causes an increase in the speed of utilization of glucose, the non-esterified fatty acids have no effect. The solution used was a complex mixture containing largely lecithin and sorbitol. It has been demonstrated that sorbitol is without effect on the function of insulin (2), but in contrast, lecithin brings about the release of insulin by the pancreas (3).

In this present work, we have studied the effect of lecithin on plasma glucose and insulin levels and on glucose assimilation in six normal dogs weighing from 24 to 33 kg fasted for 18 hours and anesthetized with sodium nembutal (30 mg/kg).

In a first attempt, we performed tests for hyperglycemia by the rapid intravenous route. Plasma glucose was measured every three minutes from the seventh minute for a half-hour. Then we perfused 125 ml per hour of a solution of soy lecithin (15 g/liter in 5% sorbitol in water). After an hour of perfusion during which plasma glucose was measured every 15 minutes, we performed a second test for hyperglycemia under conditions identical to the first. The perfusion of lecithin was continued during the second hyperglycemia test (Figure 1).

In a second trial, we perfused the same lecithin solution for 90 minutes. Plasma glucose and insulin levels were measured after fasting and at 5, 10, 30, 60, and 90 minutes of the perfusion and 15 minutes after it was stopped.

Plasma glucose was measured in heparinized and fluorinated blood with a Technicon autoanalyzer according to an adaptation of Hoffman's method (4).

- (1) E. Couturier and V. Conard, C.R. Soc. Biol., 1969, v. 163, p. 225.
- (2) W. Montague, S.L. Howell and K.W. Taylor, Nature, 1967, v. 215, p. 1088.
- (3) W. Malaisse and F. Malaisse-Lagae, J. Lab. Clin. Med., 1968, v. 72, p. 438.
- (4) W.S. Hoffman, J. Biol. Chem., 1937, v. 51, p. 120.

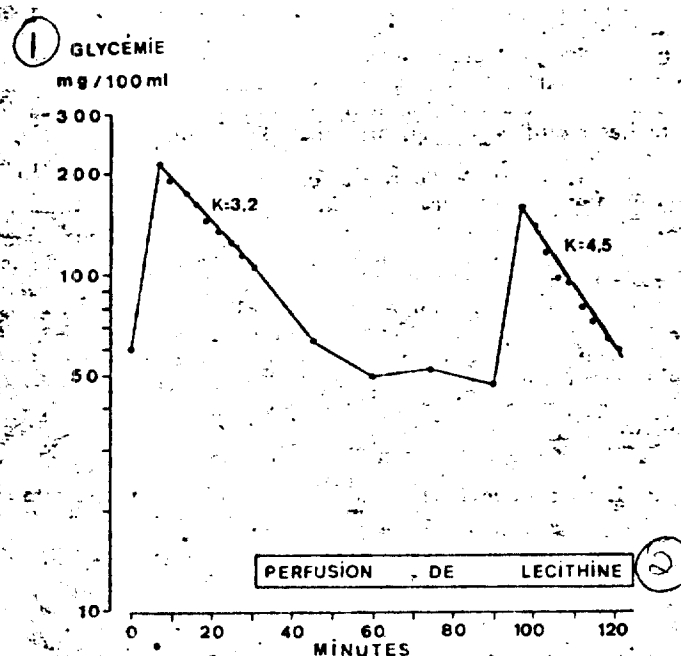


Figure 1. Effect of Lecithin Perfusion on Glucose Assimilation in Normal Dogs

1 - Glycemia

2 - Lecithin Perfusion

The insulin in the plasma was measured twice by an immunological method using two antibodies (5). The calculation of the coefficient of glucose assimilation K was done according to Conard's method (6).

The coefficient of glucose assimilation measured during the perfusion of lecithin is significantly higher than at fasting:

$$K_1 = 3.2 \pm 0.4\% \text{ per minute}; \quad K_2 = 4.5 \pm 0.7\% \text{ per minute} \quad (P < 0.01) \quad (\text{Figure 1})$$

In the course of the lecithin perfusion glycemia progressively diminished (Figure 2).

Only the levels of plasma glucose at the 90th minute of the perfusion and at 15 minutes after it was stopped are significantly less ($P < 0.005$) than the base levels. The value at fasting is $19 \pm 2 \mu\text{U/ml}$; at 30 minutes: $27 \pm 2 \mu\text{U/ml}$; at 90 minutes: $18 \pm 4 \mu\text{U/ml}$ (Figure 2). A control perfusion of physiological serum brought about no change in glycemia and a control perfusion of sorbitol did not change the coefficient of glucose assimilation: $K_1 = 4.6$; $K_2 = 4.7\%$ per minute.

(5) C.R. Morgan and A. Lazarow, Diabetes, 1963, v. 12, p. 115.

(6) V. Conard, Acta Medica Belgica, Brussels, 1955.

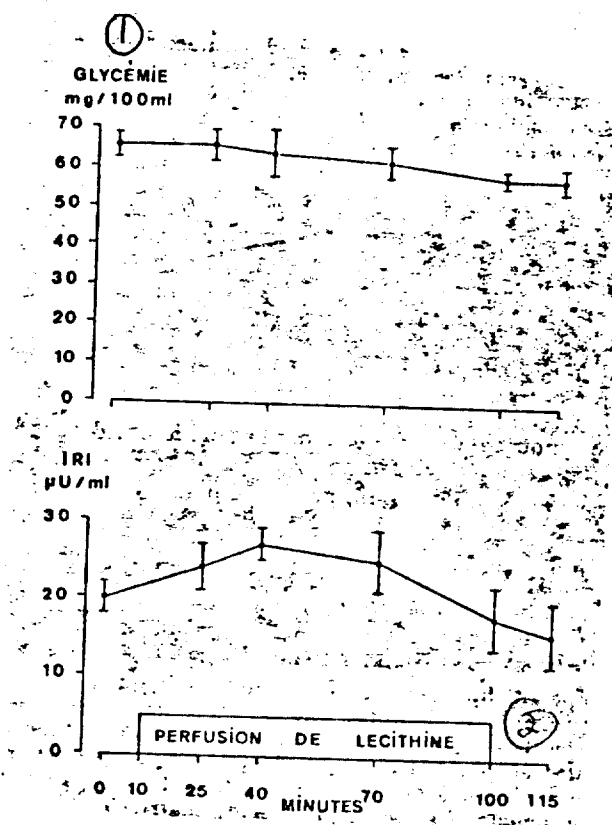


Figure 2. Effect of Lecithin Perfusion on Plasma Insulin and Glucose in Normal Dogs ()

1 - Glycemia

2 - Lecithin Perfusion

In conclusion, under the experimental conditions used, lecithin brought about an increase in plasma insulin levels, a fall in plasma glucose and a significant increase in glucose assimilation.

These observations in vivo confirm the data obtained with rat pancreas fragments incubated in vitro. The presence of lecithin in injected triglyceride emulsions is likely to modify the various evidences of glucose metabolism.

(Laboratory of Experimental Medicine and Pathological Physiology, Faculty of Medicine of the Free University of Brussels, Belgium)

Physiologie.

C. R. Soc. Biol. 1969, 163(2) 545-8

**Action de la lécithine sur le métabolisme glucidique
du chien normal.**

par E. COUTURIER et V. CONARD.

Dans un article antérieur, nous avons étudié l'influence des triglycérides et des acides gras non estérifiés plasmatiques sur la glycémie, l'insulinémie et la vitesse d'utilisation du glucose du chien normal anesthésié (1').

L'élévation des lipides est obtenue en perfusant une solution de triglycérides accompagnée ou non d'injection d'héparine. Alors que l'augmentation des triglycérides provoque un accroissement de la vitesse d'utilisation du glucose, les acides gras non estérifiés sont sans effet. La solution utilisée était un mélange complexe comportant notamment de la lécithine et du sorbitol. Il est démontré que le sorbitol est sans action sur la fonction insulinique (2'), par contre, *in vitro* la lécithine entraîne une libération d'insuline par le pancréas (3').

(1') E. Couturier et V. Conard, *C. R. Soc. Biol.*, 1969, t. 163, p. 225.

(2') W. Montague, S. L. Howell et K. W. Taylor, *Nature*, 1967, t. 215, p. 1088.

(3') W. Malaisse et F. Malaisse-Lagae, *J. Lab. Clin. Med.*, 1968, t. 72, p. 438.

Dans le présent travail nous avons étudié l'effet de la lécithine sur la glycémie, l'insulinémie et l'assimilation glucidique chez six chiens normaux pesant de 24 à 33 kg, à jeun depuis dix-huit heures et anesthésiés au nembutal sodique (30 mg/kg).

Dans un premier temps nous avons pratiqué une épreuve d'hyperglycémie par voie intraveineuse rapide. La glycémie est mesurée de 3 en 3 minutes à partir de la 7^e minute pendant une demi-heure. Ensuite on perfuse à raison de 125 ml par heure une solution de lécithine de soya (15 g/litre dans du sorbitol à 5 % en eau). Après une heure de perfusion pendant laquelle la glycémie est dosée de 15 en 15 minutes,

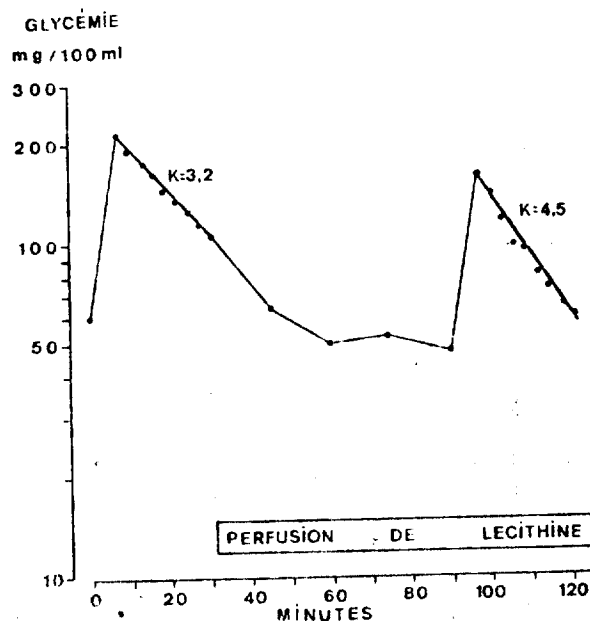


Fig. 1. — Action d'une perfusion de lécithine sur l'assimilation glucidique du chien normal.

on pratique une deuxième épreuve d'hyperglycémie dans des conditions identiques à la première. La perfusion de lécithine continue pendant la deuxième épreuve d'hyperglycémie (fig. 1).

Dans un deuxième temps nous avons perfusé la même solution de lécithine pendant 90 minutes. La glycémie et l'insuline plasmatique sont dosées à jeun puis à la 5^e, 10^e, 30^e, 60^e et 90^e minute de la perfusion et 15 minutes après l'arrêt de celle-ci.

La glycémie est dosée sur sang hépariné et fluoré à l'autoanalyseur Technicon selon une adaptation de la méthode de Hoffman (4^e). L'in-

(4^e) W. S. Hoffman, *J. Biol. Chem.*, 1937, t. 51, p. 120.

suline est dosée en double sur plasma par une méthode immunologique à deux anticorps (5*). Le calcul du coefficient d'assimilation glucidique K est fait selon la méthode de Conard (6*).

Le coefficient d'assimilation glucidique mesuré au cours de la perfusion de lécithine est significativement plus élevé qu'à jeun : $K_1 = 3,2 \pm 0,4$ % par minute ; $K_2 = 4,5 \pm 0,7$ % par mn ($P < 0,01$) (fig. 1).

Au cours d'une perfusion de lécithine la glycémie s'abaisse progressivement (fig. 2). Seules les glycémies à la 90^e minute de la perfusion et

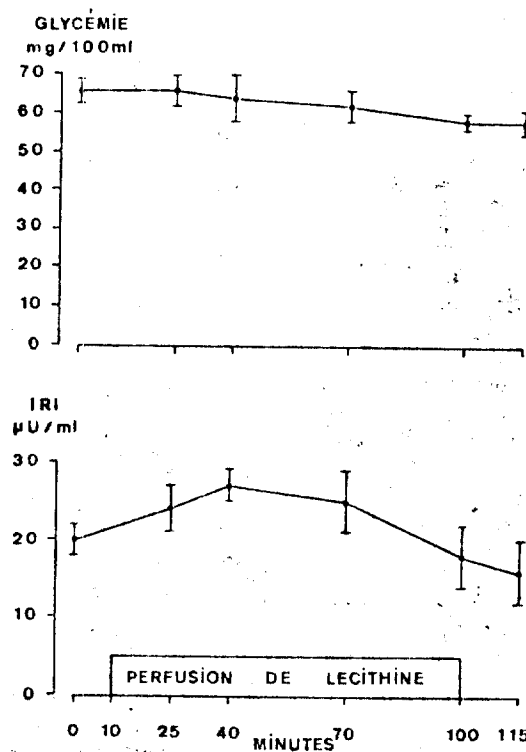


Fig. 2. — Action d'une perfusion de lécithine sur l'insulinémie et la glycémie du chien normal.

à la 15^e minute après son arrêt sont significativement inférieures ($P < 0,005$) aux taux de base. L'insulinémie s'élève significativement à la 30^e minute de la perfusion ($P < 0,005$). La valeur à jeun est de 19 ± 2 μU/ml ; à la 30^e minute : 27 ± 2 μU/ml ; à la 90^e minute : 18 ± 4 μU/ml (fig. 2). Une perfusion contrôlée de sérum physiologique

(5*) C. R. Morgan et A. Lazarow, *Diabetes*, 1963, t. 12, p. 115.

(6*) V. Conard, *Acta Medica Belgica*, Bruxelles, 1955.

n'entraîne pas de modification de la glycémie et une perfusion contrôlée de sorbitol ne modifie pas le coefficient d'assimilation glucidique : $K_1 = 4,6$; $K_2 = 4,7$ % par mn.

En conclusion, dans les conditions expérimentales utilisées la lécithine entraîne un accroissement de l'insulinémie, une chute de la glycémie et un accroissement significatif de l'assimilation du glucose.

Les observations *in vivo* confirment les données obtenues sur des fragments de pancréas de Rat incubés *in vitro*. La présence de lécithine dans les émulsions de triglycérides injectés est susceptible de modifier les différents témoins du métabolisme glucidique.

(Laboratoire de Médecine expérimentale et de Physiologie pathologique,
Faculté de Médecine de l'Université libre de Bruxelles, Belgique).

Jour. Pharmacol. And Exp. Therapy 79(1)37-41

THE EFFECT OF OXYGEN, SOYBEAN LECITHIN, CARBAMYL
CHOLINE, AND FURFURYL TRIMETHYL AMMONIUM
IODIDE ON EXPERIMENTAL POLYCYTHEMIA¹

JOHN EMERSON DAVIS

*From the Department of Physiology and Pharmacology, University of Arkansas School of
Medicine, Little Rock*

Received for publication May 25, 1943

In previously reported experiments (1-4) we have shown that raw liver, choline, certain choline esters and ethers and certain vasodilator drugs are capable of depressing experimental polycythemia in dogs and rabbits. The methods used for inducing polycythemia included daily exposure of animals to low atmospheric pressure, daily physical exercise, administration of cobaltous chloride, ephedrine or benzedrine. The depression of polycythemia is apparently unaccompanied by any dilution of the blood, and it occurs in splenectomized as well as in normal animals. The depressant drugs probably act by producing vasodilation and thereby increasing the blood and oxygen supply to red bone marrow. We have performed the reverse experiment of inducing polycythemia by the continued daily administration of vasoconstrictor drugs (ephedrine, epinephrine and posterior pituitary) to dogs and rabbits (5-6).

Theoretical considerations would indicate that if our concept of the mechanism of polycythemia depression is correct, we should be able to depress polycythemia by increasing the oxygen content of the arterial blood. The investigation herein reported shows the results of the administration (by inhalation) of an atmosphere of pure oxygen to polycythemic dogs for one hour daily. We also report the results of the daily administration of soybean lecithin, carbaminoyl choline and furfuryl trimethyl ammonium iodide to polycythemic dogs.

PROCEDURE. Experimental polycythemia was produced in three dogs by the daily subcutaneous administration of five units of solution of posterior pituitary or pitressin, and in two dogs by the daily oral administration of three mgm. per kgm. of cobalt as the chloride salt. Two of the dogs had been splenectomized 6 months prior to the commencement of these experiments. All animals were maintained on a constant adequate diet, and were allowed water *ad libitum*.

After the establishment of polycythemia, which required two or three weeks, 3 dogs receiving pituitrin and 2 dogs receiving cobalt were given commercial soybean lecithin² in a daily oral dose of 3 grams.

Subsequently, carbaminoyl choline chloride³ was administered subcutaneously to 4 polycythemic dogs in a total daily dose of 0.1 mgm. Fumethide (furfuryl trimethyl

¹ Research paper No. 535, journal series, University of Arkansas.

² Commercial Soybean lecithin was generously supplied by the American Lecithin Co., Elmhurst, L. I., N. Y.

³ Carbaminyl choline chloride (Doryl) was provided by Merck and Co. of Rahway, N. J.

ammonium iodide)⁴ was administered to 3 dogs with pituitrin-induced polycythemia in a daily subcutaneous dose of 5 mgm. (about 0.5 mgm. per kgm.).

Pure oxygen was administered for one hour daily to 3 dogs having pituitrin-induced polycythemia. The animals breathed the oxygen in a closed system consisting of a tight nosepiece connected with a Sanborn basal metabolism apparatus. The expired carbon dioxide was absorbed by soda lime, while the consumed oxygen was replaced by a steady flow of the pure gas into the metabolism machine.

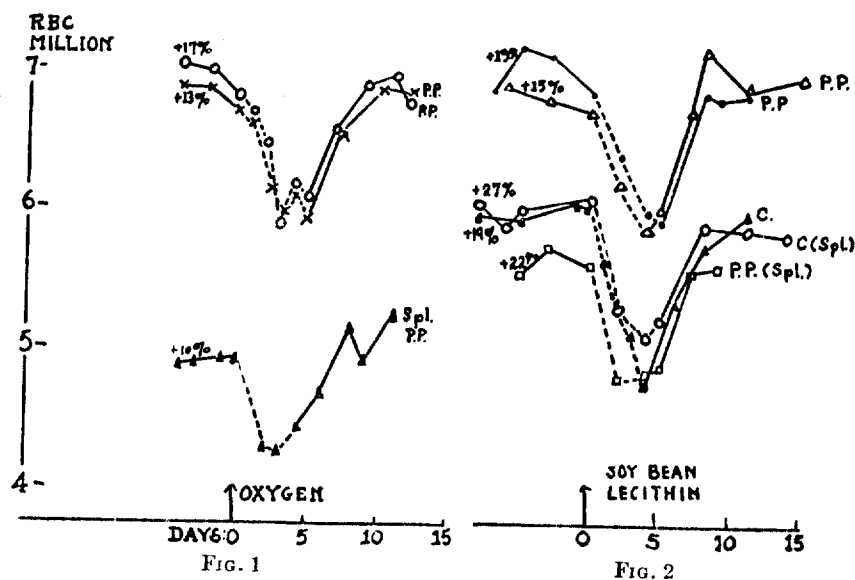


FIG. 1 (left). DEPRESSION OF PITUITRIN-INDUCED POLYCYTHEMIA IN 3 DOGS, BY THE INHALATION OF 100% OXYGEN FOR ONE HOUR DAILY

Dashed lines indicate oxygen administration. Figures at start of each line indicate the degree of polycythemia for each animal in terms of percentage increase of erythrocyte count above normal. Spl. = splenectomized dog.

FIG. 2 (right). EFFECT OF DAILY ORAL ADMINISTRATION OF 3 GRAMS OF SOYBEAN LECITHIN ON EXPERIMENTAL POLYCYTHEMIA

P.P. = dog receiving posterior pituitary injections; C = cobalt fed dog.

Blood samples were drawn from the external saphenous veins of the dogs while they were blindfolded and in a quite, unexcited state, at least 18 hours after any previous experimental procedure or medication. Red cell counts and hemoglobin percentages (Hellige) were determined at frequent intervals throughout the experiments, while total leukocyte counts were made occasionally.

RESULTS. The administration by inhalation of pure oxygen to 3 polycythemic dogs for one hour daily caused prompt reductions of their erythrocyte counts (figure 1) and hemoglobin percentages, in spite of continued daily injections of pituitrin or pitressin. The depression of the polycythemia persisted for the 5 or 6 days during which 100% oxygen was administered, and cessation of oxygen administration was followed in 3 or 4 days by a return of the polycythemia.

⁴Furmethide was supplied by the Smith, Kline, and French Laboratories, Philadelphia, Pa.

Total leukocyte counts remained fairly constant throughout the experiment. One of the animals had been splenectomized six months prior to this investigation.

Figure 2 shows the results of the daily oral administration of 3 grams of soy-bean lecithin to 2 dogs having cobalt polycythemia and 3 dogs with pituitrin-induced polycythemia. It will be noted that this procedure caused gradual reductions in the red cell counts of all 5 dogs, which persisted throughout the period of lecithin feeding in spite of continued hemopoietic stimulation by cobalt or pituitrin. Hemoglobin percentages (not shown) varied proportionately with the erythrocyte counts, but leukocyte counts showed no uniform change and remained fairly constant. Upon cessation of lecithin administration, poly-

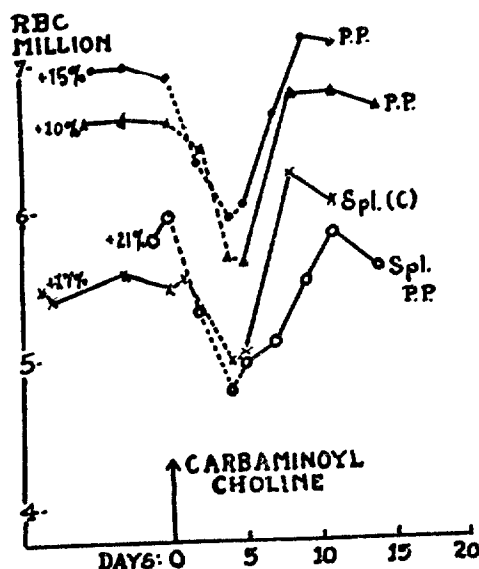


FIG. 3. DEPRESSION OF POLYCYTHEMIA BY CARBAMINOYL CHOLINE CHLORIDE

Dashed lines indicate time during which drug was administered. Figures at left of each line show degree of polycythemia for each animal as per cent increase of erythrocytes over normal.

P.P. = posterior pituitary injected dogs; C = cobalt fed dog; Spl. = splenectomized dog

cythemia returned to all dogs within about 4 days. Two of the dogs used in this experiment had been splenectomized previously.

The subcutaneous administration of a total daily dose of 0.1 mgm. of carbaminoyl choline chloride to 3 dogs with pituitrin-induced polycythemia and one cobalt-fed dog produced significant depressions in their red cell counts (figure 3) and hemoglobin percentages within 4 days. Upon cessation of "Doryl" injections, the erythrocyte numbers of these animals gradually returned to their polycythemic levels. Leukocyte counts remained fairly constant throughout these procedures. Two of the dogs had been previously splenectomized.

One splenectomized and two normal dogs having pituitrin-induced polycythemia were given daily subcutaneous injections of furothide (furfuryl tri-

methyl ammonium iodide). As will be seen in figure 4, this procedure caused a prompt diminution in the erythrocyte numbers of all 3 dogs, in spite of continued injections of posterior pituitary. Upon cessation of "Furmethide" administration, the red cell counts of all dogs returned gradually to polycythemic levels. A dose of 10 mgm. (1 mgm. per kgm.) injected into one dog resulted in death preceded by vomiting, urination, defecation and muscle twitching.

Discussion. The observation that the breathing of an atmosphere of pure oxygen by polycythemic dogs causes a depression of their erythrocyte counts (figure 1) strongly supports our theory that raw liver and vasodilator drugs

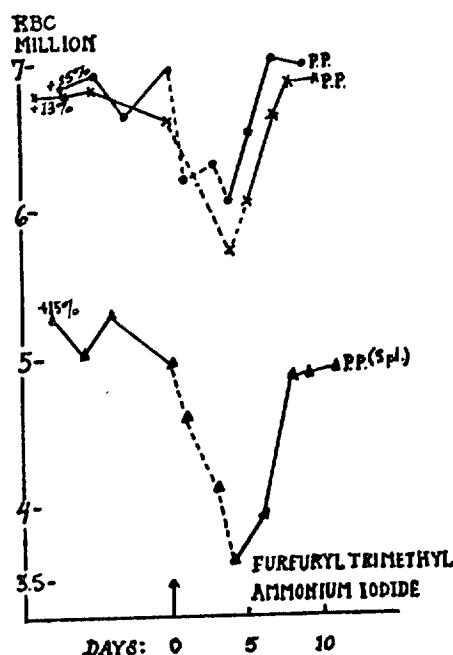


FIG. 4. EFFECT OF FURFURYL TRIMETHYL AMMONIUM IODIDE UPON THE RED CELL COUNTS OF 3 POLYCYTHEMIC DOGS

Figures at left of each line give the degree of polycythemia in each animal, before depression by the drug. Dashed lines indicate duration of "Furmethide" administration. Spl. = splenectomized animal.

depress polycythemia by increasing the oxygen supply to bone marrow (1-4). This seems especially probable because the depression of erythrocyte counts requires about the same time (i.e., 3 or 4 days) whether it is accomplished by oxygen or vasodilator drugs (figures 1 and 3). Boothby, Mayo, and Lovelace (7) have reported that the administration of pure oxygen to normal individuals is capable of increasing the oxygen content of their arterial blood by as much as 15%. Presumably an increased oxygen content in the arterial blood of our polycythemic dogs was sufficient to restore to normal the oxygen supply to their bone marrow, which had probably been curtailed by a reduction of blood supply elicited by the vasoconstrictor action of posterior pituitary injection.

Commercial soybean lecithin, which reduced polycythemia in our experiments (figure 2), probably did so by virtue of its choline content which is reported to be about 3% (8) presumably in an available form. We have previously reported that purified egg lecithin in a daily oral dose of 1 gram is without effect on experimental polycythemia (3).

It is not surprising that carbamyl choline chloride depressed polycythemia in our dogs, since we have shown previously (3, 4) that choline and acetyl beta-methyl choline, which also produce vasodilation, are effective against polycythemia.

Furfuryl trimethyl ammonium iodide was found by Fellows and Livingston (9) to exert parasympathomimetic actions, some of which are blocked by atropine. Since it was shown to produce vasodilation, we tried it on our polycythemic dogs and found that it was capable of reducing their red cell counts (figure 4).

It should be emphasized that total leukocyte counts showed no constant change either during the production or depression of polycythemia, and that splenectomized dogs responded in exactly the same manner as normal dogs.

CONCLUSIONS

Exposure for one hour daily to an atmosphere of 100% oxygen caused significant reductions in the basal erythrocyte numbers of 3 dogs with pituitrin-induced polycythemia in spite of continued daily injections of pituitrin. One of the animals had been splenectomized long before this experiment.

The daily oral administration of 3 grams of soybean lecithin to 5 polycythemic dogs caused significant reductions in their erythrocyte counts, with no appreciable change in leukocyte counts, in spite of continuation of hemopoietic stimulating measures.

Carbamyl choline chloride, in a daily subcutaneous dose of 0.1 mgm., depressed experimental polycythemia in 2 splenectomized and 2 normal dogs in spite of continued daily procedures used to produce the polycythemia (i.e., cobalt or pituitrin administration).

Furfuryl trimethyl ammonium iodide likewise depressed "pituitrin polycythemia."

These experimental results indicate that the agents used to depress polycythemia probably act by increasing the oxygen supply to the red bone marrow, thereby diminishing the local hypoxia which is probably the stimulus to the development of polycythemia.

REFERENCES

- (1) DAVIS, *Am. J. Physiol.*, **122**: 397, 1938.
- (2) DAVIS, *Ibid.*, **127**: 322, 1939.
- (3) DAVIS, *THIS JOURNAL*, **70**: 408, 1940.
- (4) DAVIS, *Ibid.*, **73**: 462, 1941.
- (5) DAVIS, *Am. J. Physiol.*, **134**: 219, 1944.
- (6) DAVIS, *Ibid.*, **137**: 600, 1942.
- (7) BOOTHBY, MAYO, AND LOVEFACE, *J. Am. Med. Assoc.*, **113**: 477, 1939.
- (8) AMERICAN LECITHIN CO. pamphlet, Soybean Lecithin as a Dietary Source of Choline.
- (9) FELLOWS AND LIVINGSTON, *THIS JOURNAL*, **68**: 231, 1940.

Am. Jour. Physiol. 142(1): 65-67, 1944
 DEPRESSION OF THE NORMAL ERYTHROCYTE NUMBER BY
 SOYBEAN LECITHIN OR CHOLINE¹

JOHN EMERSON DAVIS

*From the Department of Physiology and Pharmacology, University of Arkansas School of
 Medicine, Little Rock*

Received for publication May 4, 1944

In 1939 we reported that choline was effective in depressing the experimental polycythemias produced in dogs by either cobalt feeding or daily exposure to low atmospheric pressure, but that the administration of choline to normal dogs for 5 to 7 days was without effect on the normal red cell count (1). Later (2) we reported that soybean lecithin was capable of reducing experimental polycythemia in dogs. The mechanism of action of both substances was postulated to be a depression of red cell production by increased blood (and oxygen) supply to bone marrow, mediated by a vasodilator action of the drugs.

The purpose of the present investigation was to learn whether the prolonged daily administration of choline or lecithin for a period in excess of 5 to 7 days would depress the red cell count in normal dogs. This possibility did not seem unlikely, since bone marrow activity has a certain momentum or inertia which often requires considerable time for a change in rate of activity to be elicited. Certainly about 7 to 21 days may be required to produce experimental polycythemia by means of various agents (3-6).

METHODS. Red cell counts and hemoglobin percentages (Hellige) were determined regularly on normal dogs which were fed an adequate diet of Purina dog chow, and rolled oats. Occasional leukocyte counts and hematocrit determinations were also made.

After adequate control determinations had been made, the dogs were fed 5 grams of commercial soybean lecithin² daily. In the choline experiments, dogs were given 8 mgm. of choline hydrochloride per kgm. of body weight daily, by stomach tube (in dilute solution).

In all cases blood samples were drawn from the saphenous veins of the dogs while they were unexcited and in a fairly basal condition, at least 18 hours after previous feeding or drug administration.

RESULTS. Figure 1 shows the red cell counts of 4 normal dogs which were fed soybean lecithin daily. It will be seen that, after a latent period of 5 or more days, the erythrocyte numbers were gradually reduced. Maximal diminutions of 15 to 20 per cent were reached after 12 to 25 days of lecithin feeding. The long dashes in the line representing one dog (fig. 1) indicate a period during which 60 grams of lard were fed daily in addition to the lecithin. Cessation of lecithin feeding (short dashes) resulted in a return of the red cell counts of all dogs to normal within 11 to 20 days.

¹ Research paper no. 548, Journal series, University of Arkansas.

² Soybean lecithin was generously furnished by the American Lecithin Company of Elmhurst, L. I., N. Y.

Figure 2 shows the erythrocyte counts of 4 dogs which received choline hydrochloride (8 mgm. per kgm.) in dilute solution by stomach tube. Two of the dogs received atropine sulfate (0.5 mgm. per kgm.) by stomach tube for 18 days (dashed lines) in addition to the choline. It will be seen that the two dogs which received choline alone showed significant reductions in erythrocyte counts after 15 days of choline feeding, while those receiving atropine in addition required about 10 days after atropine cessation, or a total of about 30 days to show comparable depressions. The atropine appears to have blocked the action of the choline. Hemoglobin and hematocrit percentages were reduced proportionately with the red cell counts by choline, but leukocyte counts did not change significantly (not shown). The return of the red blood cell counts to normal is not shown because it was desired to continue these dogs on a somewhat related experiment.

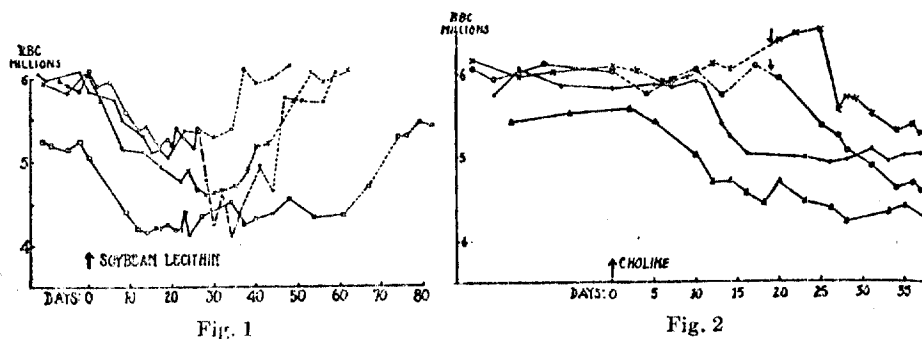


Fig. 1. The effect of soybean lecithin on the red blood cell counts of four normal dogs. Dashes indicate cessation of lecithin administration. Long dashes in solid dot line indicate period during which 60 grams of lard was fed daily, in addition to the lecithin (5 grams daily).

Fig. 2. The effect of choline hydrochloride on the erythrocyte numbers of four normal dogs. Dashes indicate periods during which 0.5 mgm. per kgm. of atropine sulfate was fed daily, in addition to the choline chloride (8 mgm. per kgm., daily).

DISCUSSION. It would seem that choline and soybean lecithin probably lower the erythrocyte number by actually *depressing erythropoiesis*, if we may judge from the slow onset of choline action (fig. 2) and the slow recovery of the red blood cell count to normal after the cessation of soybean lecithin feeding (fig. 1). It is difficult to believe that other possible mechanisms such as hemolysis, blood dilution, or sequestration of erythrocytes in blood reservoirs could be concerned, —since they should not require 10 or more days to reduce the erythrocyte count to the extent shown in these experiments.

The time required for the mild anemia to develop in these experiments is comparable to that required in previous experiments to induce experimental polycythemia by various means (3-6). It seems highly possible that in both cases we are changing the rate of erythrocyte production—but in opposite directions.

The action of choline is a muscarinic action since it is antagonised by atropine in these experiments (fig. 2). Previously, we have shown that choline depressed

polycythemia probably by a vasodilator action, because its action was blocked by atropine, and because such vasodilators as nitrites and aminophylline were also effective in reducing certain experimental polycythemias (7, 8). Vasodilator drugs may depress erythropoiesis by improving the blood flow, and consequently increasing the oxygen supply, to bone marrow. Indeed, we have also approached this problem by increasing the *oxygen content* of arterial blood. We have depressed pituitrin-induced polycythemia in dogs by the administration of 100 per cent oxygen (by inhalation) for one hour daily (2).

As indicated in figure 1 by the long dashes, one dog apparently showed an extra reduction in erythrocyte count due to the feeding of fat (lard). It must be admitted, however, that three other dogs showed no decrease of erythrocytes when similarly treated. Our purpose in testing the effect of fat-feeding was to try to support the observations of Johnson et al. (9, 10) who, while unable to demonstrate a change in the erythrocyte number, have shown that the feeding of fat increases red cell fragility and causes increased erythrocyte destruction as judged by increased bile pigment output.

We assume that soybean lecithin probably affects the erythrocyte count by virtue of its choline content, which is placed at about 3 per cent (11).

CONCLUSIONS

The daily oral administration of 5 grams of soybean lecithin to 4 normal dogs caused significant reductions in their red blood cell counts, which persisted for at least 10 days after cessation of lecithin feeding.

Choline hydrochloride, in a daily oral dose of 8 mgm. per kgm. of body weight, caused significant depressions in the erythrocyte numbers of 4 dogs. More than ten days were required for maximum depression of red cells to occur, and atropine was shown to block the depression for a longer period than the 18 days during which it was administered.

These results are explained by assuming that choline depresses erythropoiesis by increasing the blood flow and oxygen supply to bone marrow—through its vasodilator action.

REFERENCES

- (1) DAVIS, J. E. *This Journal* **127**: 322, 1939.
- (2) DAVIS, J. E. *J. Pharmacol. and Exper. Therap.* **79**: 37, 1943.
- (3) DAVIS, J. E. *Proc. Soc. Exper. Biol. and Med.* **37**: 96, 1937.
- (4) DAVIS, J. E. *This Journal* **129**: 140, 1940.
- (5) DAVIS, J. E. *This Journal* **134**: 219, 1941.
- (6) DAVIS, J. E. *This Journal* **137**: 699, 1942.
- (7) DAVIS, J. E. *J. Pharmacol. and Exper. Therap.* **70**: 408, 1940.
- (8) DAVIS, J. E. *J. Pharmacol. and Exper. Therap.* **73**: 162, 1941.
- (9) LONGINI AND JOHNSON. *This Journal* **140**: 349, 1943.
- (10) LOEWY, FREEMAN, MARCHELLO AND JOHNSON. *This Journal* **138**: 230, 1943.
- (11) Amer. Lecithin Co. pamphlet, Soybean lecithin as a dietary source of choline.

Lecithin in Experimental Arteriosclerosis

(A Preliminary Study)

by

WM. G. DOWNS, JR.

CHICAGO, ILL.

IT HAS been known for several years that a continuing state of cholesterinemia would produce a type of change in the intima of blood vessels, and extending into the media, not greatly differing from the changes found at autopsy in arteriosclerotic individuals. It would seem from this that faulty fat metabolism might be one factor of importance in the causation of this most common morbid condition.

With this in mind the following studies, of which this is a preliminary report, were instituted. Young, adult rabbits were put in quarantine for a ten-day period and standardized on an as nearly as possible, identical control diet of whole oats, carrots and lettuce. They were then divided into dietary sub-groups as follows: groups of two animals each, received in addition to their basic diet the following: $\frac{1}{4}$, $\frac{1}{2}$, 1, and 2 grams of cholesterol daily respectively, other groups 3, 6, 12 and 18 grains of pure lecithin daily respectively, and others 2, 4, 6 and 8 minims of vitamin D concentrate (250D) daily respectively. In additional groups these elements were combined in varying proportions, so that each possible combination of any two or all three of these ingredients were included in the diet of one sub-group of two animals.

It is unnecessary to detail these experiments further at this time as they will be published in full later. In the animals receiving from $\frac{1}{2}$ grain of cholesterol daily up to the maximum dosage, fairly typical

changes in the intima and inner coats of the media began to appear in four weeks. These changes did not appear sooner, but continued at a more rapid rate in the animals at the higher levels of cholesterol feeding. It was likewise somewhat accentuated by the addition of the vitamin D concentrate.

In the case of the animals receiving lecithin only, however, at the lower "feeding levels" no change was noted, while in the higher levels, the animals reacted strongly and the maximum doses could not be continued for more than a few days.

It was in the groups in which the lecithin dosage was small, but with the addition of cholesterol and vitamin D, that the most interesting findings were made. Here, in animals receiving lecithin plus cholesterol alone or lecithin plus cholesterol plus vitamin D, it was still not possible to produce vascular changes. This was true where the dosage of cholesterol and vitamin D were at the highest levels administered and over a period of four months.

It would seem that the addition of relatively small quantities of lecithin to the diet, will prevent the occurrence of the experimental arteriosclerosis of high cholesterol diet origin. Further studies are now in progress, to determine the effects, if any, of lecithin on experimental arteriosclerosis from other causes than cholesterinemia.

Chicago Medical School.
July 10, 1933.

LAURYL PEROXIDE - LECITHIN

L PEROXIDE

CLEARED FOR USE AS A POLYMERIZATION CATALYST IN PAPER AND PAPERBOARD FOR AQUEOUS AND FATTY FOOD USE UNDER §121.2526 (COMPONENTS OF PAPER AND PAPERBOARD IN CONTACT WITH AQUEOUS AND FATTY FOODS).

CLEARED FOR USE AS A POLYMERIZATION CATALYST IN PAPER AND PAPERBOARD FOR DRY FOOD CONTACT UNDER §121.2571 (COMPONENTS OF PAPER AND PAPERBOARD IN CONTACT WITH DRY FOOD).

LAURYL PYRIDINIUM 5-CHLORO-2-MERCAPTOBENZOTHAZOLE

CLEARED FOR USE IN ADHESIVES UNDER §121.2520 (ADHESIVES).

LAURYL SULFATE SALTS

THE AMMONIUM, MAGNESIUM, POTASSIUM, AND SODIUM SALTS OF LAURYL SULFATE CLEARED FOR USE UNDER §121.2507 (CELLOPHANE); AND UNDER §121.2567 (WATER-INSOLUBLE HYDROXYETHYL CELLULOSE FILM). (FR SEPT. 12, 1964).

LAURYL SULFATE SALTS OF AMMONIUM, MAGNESIUM, POTASSIUM, AND SODIUM CLEARED FOR USE IN ADHESIVES UNDER §121.2520 (ADHESIVES).

AMMONIUM, MAGNESIUM, POTASSIUM, AND SODIUM SALTS OF LAURYL SULFATE CLEARED FOR USE IN PAPER AND PAPERBOARD FOR AQUEOUS AND FATTY FOOD USE UNDER §121.2526 (COMPONENTS OF PAPER AND PAPERBOARD IN CONTACT WITH AQUEOUS AND FATTY FOODS).

AMMONIUM, POTASSIUM, SODIUM, AND MAGNESIUM SALTS OF LAURYL SULFATE CLEARED FOR USE IN PAPER AND PAPERBOARD FOR DRY FOOD CONTACT UNDER §121.2571 (COMPONENTS OF PAPER AND PAPERBOARD IN CONTACT WITH DRY FOOD).

LAVANDIN (HYBRIDS BETWEEN LAVANDULA OFFICINALIS CHAIX AND LAVANDULA LATIFOLIA VILL.)

GENERALLY RECOGNIZED AS SAFE AS A NATURAL FLAVOR EXTRACTIVE UNDER §121.101.

LAVENDER (LAVANDULA OFFICINALIS CHAIX.)

GENERALLY RECOGNIZED AS SAFE AS A NATURAL FLAVORING AND NATURAL FLAVOR EXTRACTIVE UNDER §121.101.

LAVENDER SPIKE (LAVANDULA LATIFOLIA VILL.)

GENERALLY RECOGNIZED AS SAFE AS A NATURAL FLAVOR EXTRACTIVE UNDER §121.101.

LEAD

FDA LETTER AFFIRMING GENERAL RECOGNITION OF SAFETY OF SOFT SOLDER CONTAINING 50% LEAD AND 50% TIN FOR USE IN DAIRY PROCESSING EQUIPMENT REVOKED APRIL 9, 1970.

LEAF ALCOHOL

CLEARED UNDER §121.1164 (SYNTHETIC FLAVORING SUBSTANCES AND ADJUVANTS).

LECHE CASPI (SORVA) (COUMA MACROCARPA BARB. RODR.)

CLEARED FOR USE AS A NATURAL (COAGULATED OR CONCENTRATED LATIC) MASTICATORY SUBSTANCE OF VEGETABLE ORIGIN IN CHEWING GUM BASE, UNDER §121.1059 (CHEWING GUM BASE).

CLEARED FOR USE IN THE FOOD-CONTACT SURFACE OF LABELS AND/OR TAPES UNDER §121.2577 (PRESSURE-SENSITIVE ADHESIVES).

LECHE DE VACA (BROSIMUM UTILE (H.B.K.) PITTIER AND POULSENIA SPP.; LACMELLEA STANDLEYI (WOODSON); MONACHINO)

CLEARED FOR USE AS A NATURAL (COAGULATED OR CONCENTRATED LATIC) MASTICATORY SUBSTANCE OF VEGETABLE ORIGIN IN CHEWING GUM BASE, UNDER §121.1059 (CHEWING GUM BASE).

CLEARED FOR USE IN THE FOOD-CONTACT SURFACE OF LABELS AND/OR TAPES UNDER §121.2577 (PRESSURE-SENSITIVE ADHESIVES).

LECITHIN

GENERALLY RECOGNIZED AS SAFE AS A MISCELLANEOUS AND/OR GENERAL PURPOSE FOOD ADDITIVE UNDER §121.101.

CLEARED FOR USE UNDER §121.2514 (RESINOUS AND POLYMERIC COATINGS), §121.2526 (COMPONENTS OF PAPER AND PAPERBOARD IN CONTACT WITH AQUEOUS AND FATTY FOODS), §121.2548 (ZINC-SILICON DIOXIDE MATRIX COATINGS), §121.2550 (CLOSURES WITH SEALING GASKETS FOR FOOD CONTAINERS), §121.2559 (XYLENE-FORMALDEHYDE RESINS CONDENSED WITH 4,4'-ISOPROPYLIDENEDIPHENOL EPICHLOROHYDRIN EPOXY RESINS), AND §121.2570 (ETHYLENE-VINYL ACETATE COPOLYMERS).

CLEARED BY THE MEAT INSPECTION DIVISION AS AN ANTIOXIDANT AND EMULSIFIER IN LARD AND SHORTENING, IN AN AMOUNT SUFFICIENT FOR THE PURPOSE; AND IN OLEOMARGARINE AS AN EMULSIFIER AT 0.5%.

Food Protection Committee. 1972
 Comprehensive GRAS Survey, daily intakes---
Lecithin and Lecithin modified with Hydrogen peroxide
 National Academy of Sciences, National Research Council,
 Washington, D.C. Table 13, Part A, pages 90-93; 241-242

Food Protection Committee. 1972
Comprehensive GRAS Survey, Usage levels---
lecithin and lecithin modified with hydrogen peroxide
National Academy of Sciences, National Research Council,
Washington, D.C. Table 2, pages 19, 49

Food Protection Committee. 1972
Comprehensive GRAS Survey, total annual poundage---
Lecithin and Lecithin modified with Hydrogen peroxide
National Academy of Sciences, National Research Council,
Washington, D.C. Table 11, Part A, pages 6; 14

Jour. Lab. and Clin. Med. 34(5): 688-698

PARENTERAL NUTRITION

VIII. THE VASODEPRESSOR ACTIVITY OF SOYBEAN PHOSPHATIDE PREPARATIONS

ROBERT P. GEYER, PH.D., DONALD M. WATKIN, M.D.,
 LEROY W. MATTHEWS, B.S., AND FREDRICK J. STARE, M.D.
 BOSTON, MASS.

IN PREVIOUS reports from this laboratory the preparation and use of emulsions of fat for intravenous nutritional purposes have been described.^{1,2} Various emulsions containing soybean phosphatides as the stabilizer have been given successfully to the rat, dog, and rabbit. It was clearly shown in these studies that fat given in this manner is well utilized for energy. More recently, studies using fat labeled with C¹⁴ have confirmed the utilization of fat in emulsions given intravenously.^{3,4}

The only adverse physiologic effects which were evident when fat emulsions were given to dogs intravenously were occasional nausea, vomiting, and excessive salivation when the initial infusions were given too rapidly and a moderate normocytic anemia⁵ if the fat infusions were continued for a period of two to three weeks or longer. The anemia rapidly disappeared, however, when the infusions were discontinued. Measurements of the blood pressure of dogs by an ordinary sphygmomanometer disclosed no significant alterations from normal. However, in a few preliminary observations in man following the intravenous injections of these fat emulsions, a fall in arterial blood pressure occasionally resulted. Before continuing with clinical studies, observations were undertaken in which the cat was used in order to obtain more information on this vasodepressor phenomenon. The experiments reported in this paper were designed to (1) determine the origin and character of the vasodepressor activity, (2) compare more rigorously the species variation with respect to the vasodepressor action, and (3) investigate possible methods of removing the vasodepressor substance.

EXPERIMENTAL

Methods.—

1. Measurement of arterial blood pressure was done in the usual manner. The carotid artery of the anesthetized (Nembutal) animal was cannulated and by means of a citrate-filled system was connected to a mercury manometer provided with a moving stylus. Recordings were made on a smoked paper drum attached to a kymograph. In many cases, concurrent pneumograph recordings were also taken.*

2. Injection of the test substance was done as follows: A glass cannula was inserted into the femoral vein and by means of a narrow-bore rubber tube was connected to a

From the Department of Nutrition, Harvard School of Public Health, and the Department of Biological Chemistry, Harvard Medical School.

Supported in part by grants-in-aid from the National Dairy Council, Chicago, Ill., The Upjohn Company, Kalamazoo, Mich., the Nutrition Foundation, Inc., New York, N. Y., the Milbank Memorial Fund, New York, N. Y., and the Cancer Research Grants Branch, National Cancer Institute, Bethesda, Md.

Received for publication, Jan. 27, 1949.

*The authors wish to express appreciation to Dr. E. M. Landis, Department of Physiology, Harvard Medical School, and Dr. J. L. Whittenberger, Department of Physiology, Harvard School of Public Health, for advice and the loan of apparatus used in these studies on blood pressure.

burette which contained either 0.9 per cent sodium chloride or 5 per cent dextrose solution. The test material was injected from a suitable syringe through a short No. 24 needle which was inserted as far as possible through the rubber tube close to the cannula. Immediately after the injection and before withdrawal of the needle, 1 to 2 ml. of the solution from the burette were allowed to run in as rapidly as possible. The syringe was then quickly withdrawn.

3. Emulsification of the test material usually was accomplished in one of the following ways: (a) stirring with a wire stirrer at 4,000 r.p.m. and 90° C. for four to seven minutes; (b) blending in the Waring Blendor at high speed and at 70 to 90° C. for one to five minutes; and (c) first blending as under (b) and then homogenizing in a high-pressure homogenizer* under nitrogen at 3,500 to 4,000 p.s.i. and 85 to 90° C. for five to fifteen minutes. Where modifications of these methods were used, the changes are indicated. Tonicity was achieved by the addition of saline or dextrose to give a final concentration of 0.9 and 5 per cent, respectively. Adjustment of pH was accomplished with disodium phosphate.

4. The fat-emulsifying properties of the various fractions of soybean phosphatides were determined in the following manner. One liter of an aqueous mixture containing 3 per cent of the phosphatide fraction, 15 per cent coconut oil, and 4.7 per cent dextrose was homogenized under high pressure by the technique previously described,⁶ but using the homogenizer referred to in the preceding paragraph. Disodium acid phosphate was used when necessary to adjust the pH. When the resulting emulsion was of fine particle size, as judged by microscopic examination, a sample was autoclaved and again examined for particle size.

Origin and Character of the Vasodepressor Action and Variation Between Species.—Preliminary blood pressure measurements on the cat indicated that for this species a marked decrease in pressure occurred when even small quantities of an emulsion (Emulsion 35)¹ containing 3 per cent of a purified soybean phospholipid fraction were injected rapidly. This emulsion contained coconut oil 15 per cent, fraction B(F-2) 3 per cent, dextrose 4.7 per cent, and water 80 per cent (weight/volume). It was readily apparent that a greatly decreased response resulted when a given dose was administered for the second time even though as much as thirty minutes elapsed between the two injections. To determine whether or not the soybean phosphatide stabilizer, fraction B-(F-2),¹ was responsible for the vasodepressor activity of the fat emulsion, a 3 per cent emulsion of this material was prepared by high-pressure homogenization, made isotonic to rat serum with dextrose, and autoclaved for fifteen minutes at 15 p.s.i. in a pressure bottle filled with nitrogen. Small quantities of this preparation were injected into a cat, and the depressor response was found to be similar to that obtained when Emulsion 35 was administered.

Blood pressure studies were undertaken using the dog, rabbit, and rat as test animals in addition to the cat. All blood pressure recordings were taken in a manner similar to that used for the cat with the exception of the rat. For this species it was necessary to use a capillary manometer and record the pressure changes visually. Neither the fat emulsion (Emulsion 35) nor the 3 per cent emulsion of the phosphatide fraction B(F-2) was effective in lowering the blood pressure of the rat, rabbit, or dog more than 15 mm. even when large quantities were injected as rapidly as possible. Table I shows a typical response obtained with the cat, dog, rabbit, and rat to the injection of a blended 6 per cent preparation of the phosphatide fraction B(F-2). It is readily apparent

*Manton Gaulin Homogenizer Model No. 75-CGD, Manton Gaulin Manufacturing Company, Inc., 44 Garden Street, Everett 49, Mass.

that the cat is far more susceptible to the depressor activity and hence in all subsequent testing for vasodepressor activity the cat was used.

TABLE I. RESPONSE OF VARIOUS SPECIES TO THE VASODEPRESSOR PRESENT IN PHOSPHATIDE FRACTIONS MADE FROM COMMERCIAL SOYBEAN PHOSPHATIDES

SPECIES	BODY WEIGHT (GM.)	MATERIAL INJECTED	VOLUME INJECTED (ML.)	WEIGHT OF FRACTION INJECTED (MG./KG. B.W.)	BLOOD PRESSURE RESPONSE	
					DECREASE (MM.)	INCREASE (MM.)
Rat	225	Blended 6% fraction B(F-2)	1.0	277	—	—
		Blended 6% fraction 70-f	2.0	554	14	—
Rabbit	3,100	Blended 6% fraction B(F-2)	2.0	39	—	4
		Blended 6% fraction 70-f	1.2	23	8	—
Dog	7,000	Blended 6% fraction B(F-2)	10.0	86	15	—
Cat	1,500	Blended 6% fraction B(F-2)	0.1	4	56	24
		Blended 1.2% fraction 70-f	0.1	0.8	46	—

Since it was evident that the phosphatide preparation B(F-2) contributed the vasodepressor activity, a number of the precursors of this fraction were investigated to determine whether the depressor was present originally in the soybeans or was introduced at some point during the process of manufacture. The following materials were tested in the cat for vasodepressor activity:

1. Freshly prepared soybean phosphatides obtained by extracting 2.5 kg. quantities of ground dried soybeans for four successive three-hour periods with 3-liter portions of boiling redistilled petroleum ether. The combined extracts were concentrated in vacuo to a volume of approximately 200 ml. and 1.2 liters of anhydrous acetone were added and the mixture was allowed to stand overnight. After decantation of the clear supernatant liquid, the residue was dissolved in 40 to 50 ml. of ether, filtered, and poured into 500 ml. of acetone. The precipitate was filtered off after standing for three hours and was then dried in vacuo at room temperature. A 1.5 per cent emulsion of this material was prepared by the stirring technique, with dextrose present for tonicity, and examined for depressor activity.

2. An autoclaved portion of the 1.5 per cent emulsion of the freshly prepared soybean phosphatides.

3. Freshly prepared soybean phosphatides which had been allowed to stand in air for four days at room temperature and which were then emulsified at a 3 per cent concentration using the stirrer technique. Dextrose was added for tonicity.

4. Fat emulsion stabilized with freshly prepared soybean phosphatides and prepared by using the homogenization procedure described previously.⁶ The emulsion contained 15 per cent refined corn oil, 2 per cent freshly prepared soybean phosphatides, 5 per cent dextrose, and water.

5. Commercial Liquid Lecithin* emulsified at a 6 per cent concentration by the blender technique with dextrose present. This emulsion was not entirely stable and, therefore, only the portion which remained in true dispersion was used in the vasodepressor tests.

6. Asolectin† (commercial soybean phosphatides) emulsified by the blender procedure at a 6 per cent concentration using dextrose for tonicity.

7. Emulsion 35.¹

8. Soybean phosphatide fraction B(F-2)¹ prepared in a 3 per cent emulsion by the high-pressure homogenizer method using dextrose for tonicity.

9. Soybean phosphatide fraction B(F-2) prepared in a 6 per cent emulsion by the high-pressure homogenizer method using dextrose for tonicity.

*Liquid Lecithin, Robinson-Wagner Company, New York, N. Y.

†Asolectin was generously supplied by Dr. Albert Scharf of Associated Concentrates, Inc., Woodside, Long Island, N. Y.

TABLE II. EFFECT OF VARIOUS SOYBEAN PHOSPHATIDE PREPARATIONS ON THE BLOOD PRESSURE OF THE CAT

NO.	MATERIAL	PER CENT OF PHOSPHATIDE FRACTION (WT./VOL.)	VOLUME INJECTED (ML.)	CAT		BLOOD PRESSURE RESPONSE*	
				NO.	WT. (KG.)	DECREASE (MM.)	INCREASE (MM.)
1	Freshly prepared soybean phosphatides	1.5	0.8	1	0.97	—	10
2	No. 1 autoclaved for 15 min. at 15 p.s.i.	1.5	0.8	1	0.97	—	18
3	No. 1 allowed to stand in air at room temperature for 4 days	3.0	0.4	1	0.97	24	—
4	No. 1 + 15% corn oil as an emulsion	2.0	0.5	2	2.30	6	—
5	Liquid lecithin	6.0	0.5	3	0.67	10	—
6	Asolectin	6.0	0.1	4	1.60	—	—
7	Fraction B(F-2) + 15% coconut oil as an emulsion—Emulsion 35	3.0	0.4	5	2.00	50	—
8	Fraction B(F-2)	3.0	0.4	5	2.00	46	—
9	Fraction B(F-2): First injection Second injection (16 min. after first injection)† Third injection (18 min. after first injection)†	6.0	1.0	8	1.60	77 18 10	— — —

*Refers only to the initial response.

†In each case the blood pressure had returned to normal prior to the injection.

These preparations were injected into cats as described earlier, and blood pressure recordings were taken. In addition, Preparation 9 was injected several successive times into a cat to test for tachyphylaxis. The results, together with pertinent data, are given in Table II. It is seen that the depressor activity is primarily in the phosphatide preparations but is not present in freshly prepared material, even after autoclaving. Repeated injections of the phosphatide material result in greatly decreased vasodepressor responses. Fig. 1 shows a typical response obtained with the cat to the repeated injection of a blended 6 per cent preparation of the phosphatide fraction B(F-2).

While the experiments already described were in progress, other experiments were concurrently undertaken for the purpose of fractionating the commercial soybean phosphatide preparation known as Asolectin and the phosphatide fraction prepared from it known as fraction B(F-2) from two standpoints; namely, (1) to prepare, if possible, a depressor-free phosphatide fraction which would still be a suitable emulsifying agent, and (2) to concentrate the vasodepressor substance(s) and determine some of its characteristics.

The initial studies were made on phosphatide fractions already on hand. These fractions had been prepared some months earlier in connection with fractionations of Asolectin designed to remove the material which had caused the granulomatous lesions reported in earlier work¹ and had been stored at -8° C. during the ensuing months. The preparation of fractions A(F-1), B(F-1), and C(F-1) have been reported.¹ Since the preparation of fractions D(F-1) and E(F-1) from B(F-1) was not given previously, it will be described here.

VASODEPRESSOR EFFECT OF SOYBEAN PHOSPHATIDE GIVEN IV.

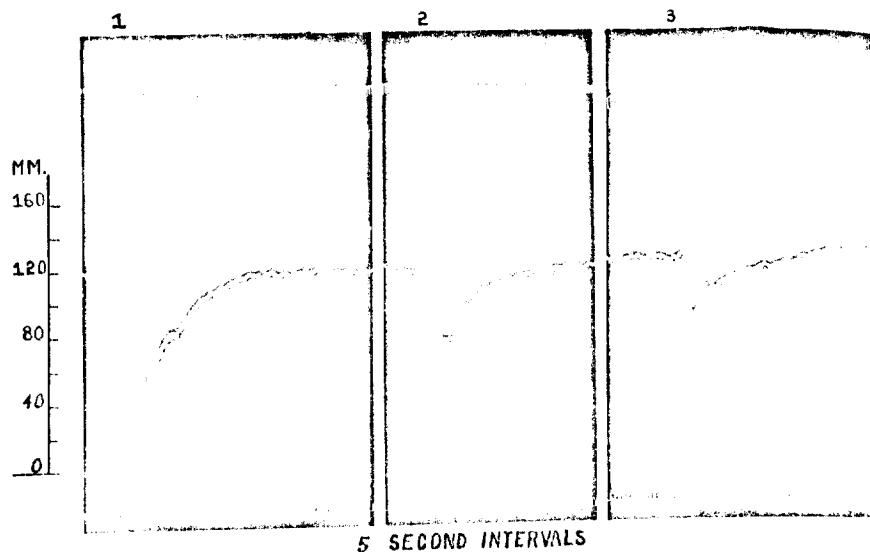


Fig. 1.—Response of blood pressure to repeated intravenous injections of 1.0 ml. of a 6 per cent emulsion of soybean phosphatide fraction B(F-2), cat No. 8 (1.6 kg.). Time between injections 1 and 2 was sixteen minutes. Time between 2 and 3 was eighteen minutes.

Preparation of D(F-1) and E(F-1): 20.5 Gm. of fraction B(F-1) were completely dissolved in 240 ml. of dry chloroform and the solution was filtered. Three hundred sixty milliliters of 95 per cent ethanol were added slowly with shaking, and the light tan precipitate which formed was filtered off, dissolved in 100 ml. of chloroform, and reprecipitated by the addition of 150 ml. of 95 per cent alcohol. This material, fraction B(F-1), was dried in vacuo and weighed 13.7 grams. The combined filtrates were concentrated to dryness in vacuo and yielded 6 Gm. of fraction D(F-1).

Each of the various fractions was blended, with dextrose present, and the vasodepressor activity was tested in the cat. Table III gives the results of these studies along with an evaluation of the emulsifying properties of the various fractions. The choline content of some of these fractions was determined by the method of Engel⁷ and is included in the table since it serves as an index of the amount of lecithins present. Lipositol prepared according to the procedure of Woolley⁸ was also included in these studies, and it was found to be no more active than the lipositol-low, alcohol-insoluble fractions.

The results obtained with all of the foregoing preparations indicated that the depressor substance was probably a degradation product of one of the phosphatides found in soybeans. Although the almost complete lack of solubility of the depressor substance in alcohol negated such compounds as free choline, ethanolamine, and many of their simple derivatives, some of these compounds were tested in the cat and the resulting effect upon blood pressure was compared with that of the unknown depressor. The character of the curve

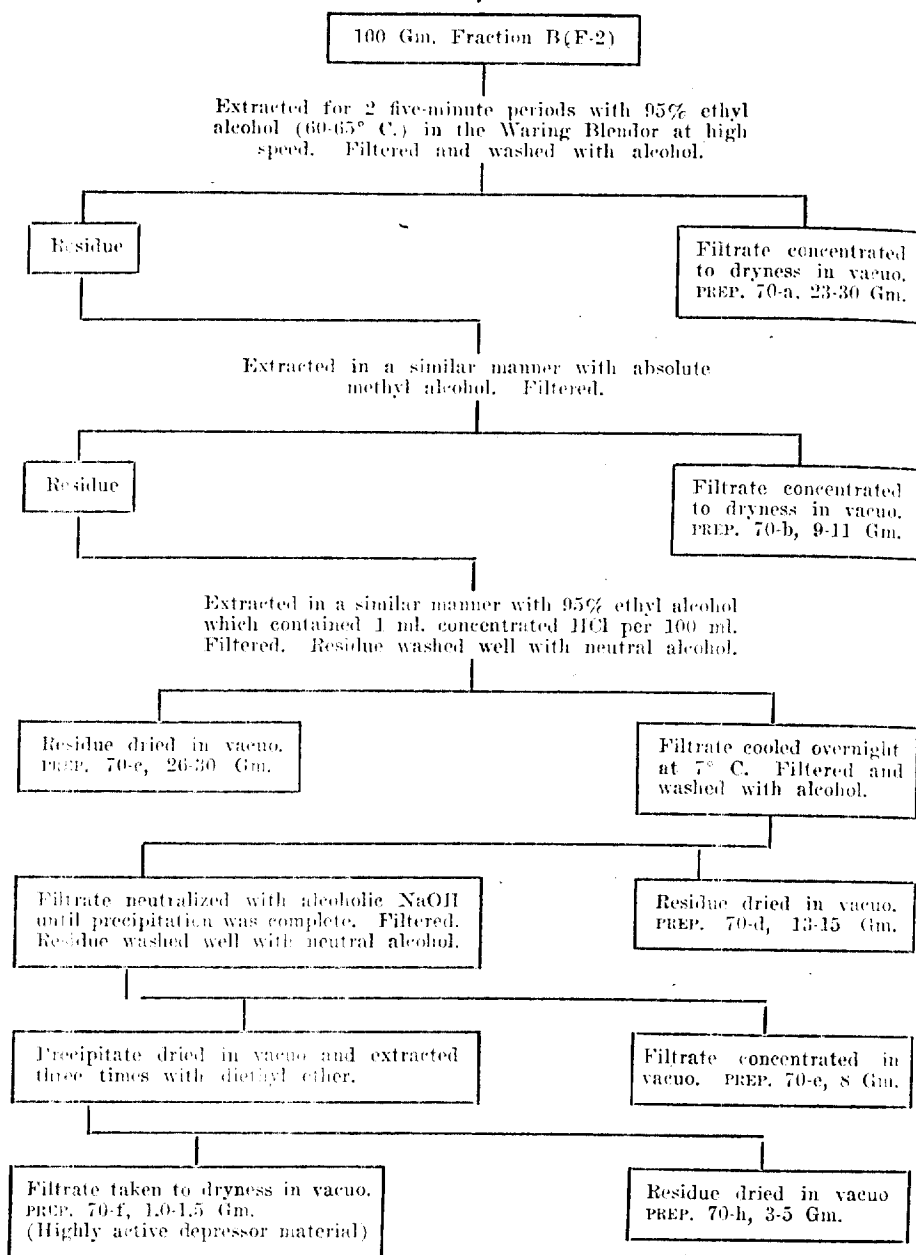
TABLE III. EFFECT OF VARIOUS FRACTIONS MADE FROM COMMERCIAL SOYBEAN PHOSPHATIDES (ASOLECTIN) ON THE BLOOD PRESSURE OF THE CAT

NO.	FRACTION	RELATIVE EMULSIFYING PROPERTIES*	CHOLINE CONTENT	CONCENTRATION PER CENT (WT./VOL.)	VOLUME INJECTED (ML.)	CAT		BLOOD PRESSURE RESPONSE	
						NO.	WT. (KG.)	DECREASE (MM.)	INCREASE (MM.)
1	A(F-1)	Poor	—	3.0	1.0	7	2.2	—	6
2	B(F-1)	Fair	0.76	3.0	0.4	7	2.2	6	—
3	C(F-1)	Very good	10.5	3.0	1.0	7	2.2	—	4
4	D(F-1)	Fair	1.85	3.0	0.4	7	2.2	6	—
5	E(F-1)	Fair	0.04	3.0	0.2	7	2.2	54	—
6	B(F-2)	Good	—	3.0	0.3	6	1.5	56	—
7	Lipositol	Poor	—	3.0	1.0	7	2.2	60	—
8	Lipositol-free residue	Fair	—	3.0	1.0	7	2.2	55	—
9	70-f	—	—	1.2	0.1	6	1.5	46	—

*Based on the rating of "good" for fraction B(F-2).

PARENTERAL NUTRITION

CHART I. FRACTIONATION OF SOYBEAN PHOSPHATIDE FRACTION B(F-2) SCHEME 71



of the blood pressure response was not similar to that of the material in the soybean phosphatide fractions, and the extreme tachyphylaxis which resulted on repeated injection of phosphatides was also absent.

The following preparations also were made and tested for depressor activity:

1. A dialysate of the fraction B(F-2) prepared by dialyzing a 6 per cent emulsion of this fraction against distilled water through a thin collodion membrane and concentrating the dialysate to the volume of the original emulsion. This material was inactive, whereas the residue was still completely active.

2. Ten milliliters of Emulsion 35 placed in a 15 ml. test tube and to which 0.5 Gm. of sodium chloride was added. The tube was heated at 70° C. for five minutes and then centrifuged while hot. The aqueous layer was tested for activity and was completely inactive.

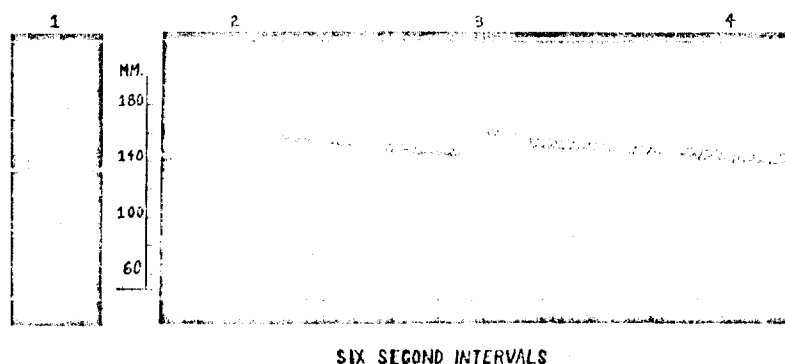


Fig. 2.—Effect on blood pressure of route of administration of soybean phosphatide fraction 70-f, cat No. 9 (2.3 kg.).

- 1, 0.1 ml. 2.5 per cent 70-f, intravenously.
- 2, 2.0 ml. 2.5 per cent 70-f, intraperitoneally.
- 3, 2.0 ml. 2.5 per cent 70-f, intraperitoneally.
- 4, 1.0 ml. 2.5 per cent 70-f, subcutaneously.

Further fractionations of the preparation B(F-2) were carried out by means of solubilities in various solvents such as ethyl alcohol, acetone, chloroform, and mixtures of these solvents, but none was effective in concentrating the depressor to a sufficient degree. Since the activity in the earlier fractionations followed those fractions high in inorganic constituents, separations were undertaken with acidic solvents. Acidic ethyl alcohol proved to be best for this purpose, and although many different fractionation schemes were carried out, only one of the most successful is described in Chart I. This fractionation of B(F-2) yields a material referred to as 70-f which is a highly active depressor substance. The results of the tests for vasodepressor activity using fraction 70-f are given in Tables I and III. The marked susceptibility of the cat to the vasodepressor activity is well shown in the comparative data in Table I.

Effect of Route of Administration (Other Than Intravenous) on Depressor Activity of Fraction 70-f.—To determine the effect of the route of administration on depressor activity, a 2.5 per cent emulsion of preparation 70-f was injected intraperitoneally into an anesthetized cat. Fig. 2 shows the results of a typical experiment, and it is apparent that instead of causing a decrease in blood pressure, a pressor response resulted. Oral administration of this preparation as well as intramuscular and subcutaneous administration was without effect. Hence the depressor effect of the phosphatide fraction is only evident when the material is given intravascularly.

Effect of the Depressor Substance on Duodenal Muscle.—To determine whether or not the depressor material had an effect on smooth muscle contractions, a strip of rat duodenal muscle was connected to a lever in the usual manner and was immersed in oxygenated mammalian Ringer's solution. At intervals 0.4 ml. of a 2.5 per cent emulsion of fraction 70-f was added; in each case the Ringer's solution was changed before the addition. Five per cent glucose solution was used as the control. The results of this experiment are

EFFECT OF DEPRESSOR MATERIAL ON SMOOTH MUSCLE CONTRACTION

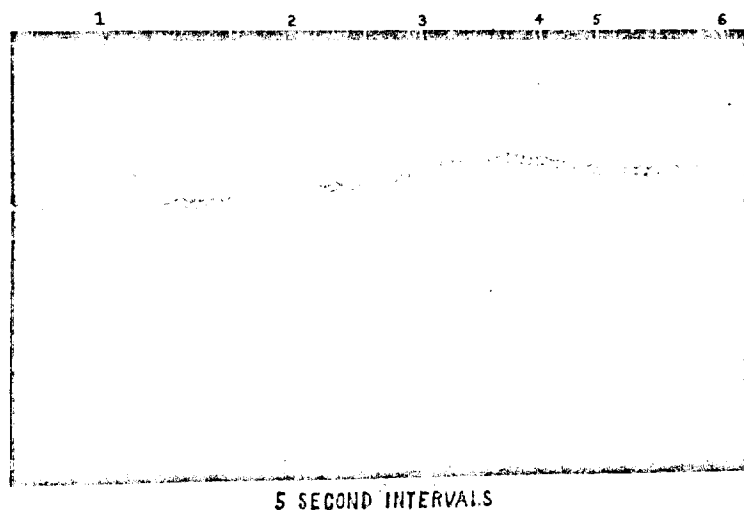


Fig. 3.—Contraction of rat duodenal muscle to successive stimulation with 0.4 ml. of an emulsion of 2.5 per cent of soybean phosphatide fraction 70-f. Volume of Ringer solution was 200 milliliters; 4 was 5 per cent dextrose solution. Time lapses between 2 and 3, 3 and 4, and 5 and 6 were 5, 6, and 5 minutes respectively.

given in Fig. 3 and it is observed that the depressor material gives rise to a marked contraction of the muscle and that the contraction decreases with repeated addition of fraction 70-f.

DISCUSSION

The soybean phosphatide fraction B(F-2) described previously¹ for use as a stabilizer in fat emulsions for intravenous nutritional purposes has been found to possess a marked vasodepressor activity when injected into the cat. This activity was present in the commercial soybean phosphatide preparation from which this fraction was obtained. Since freshly prepared soybean phosphatides had little such activity, vasodepressor substance probably is formed sometime during or after the process of manufacture by oxidative or degradative changes. This view is supported by the fact that freshly prepared phosphatides became active depressants when allowed to stand at room temperature in contact with air.

The great species difference with respect to this vasodepressor activity was of interest. In the cat, 4 mg. of fraction B(F-2) per kilogram of body weight caused a decrease in arterial blood pressure of 56 millimeters. Little or no

effect was obtained, however, when 277, 39, and 86 mg. quantities per kilogram of body weight were given to the rat, rabbit, and dog, respectively. This difference is important in work concerning intravenous fat emulsions because the dog and rat are the animals usually used for testing such preparations. From clinical studies it appears that man is more sensitive to the vasodepressor than these three species, but less so than the cat. It is obvious that it is desirable to test fat emulsions intended for clinical use in several species including the cat.

Several interesting characteristics of the pattern of the blood pressure response to the various phosphatide fractions have been observed. One of these was the step-like manner in which the blood pressure returned to normal. This ranged all the way from a slight pause or dip to a drop back to the original low. In the latter case, the return proceeded without any sign of another stepwise ascent. Such a step phenomenon is not characteristic of the response to such compounds as acetylcholine, histamine, etc. Another difference between the soybean depressant and the other depressant materials tested was the relatively great tachyphylaxis that occurred following the initial response when an original drop of more than 10 to 20 mm. had occurred. This tachyphylaxis made the exact quantitative evaluation of the potency of various preparations difficult, but a fair comparison was still possible by allowing a suitable time lapse between injections and by using a standard injection of an emulsion of the fraction B(F-2) as an index of the tachyphylaxis.

The vasodepressor substance was not one of the known phosphatides or their simple degradation products such as free ethanolamine. It was nondialyzable through collodion and insoluble in water, alcohol, and aqueous acid, but soluble in acidified alcohol. The use of the latter solvent, especially when hot, may have resulted in various extraneous cleavages, but no apparent increase in total original depressor potency occurred through its use. Perhaps the acid functioned by removing inorganic ions from the depressor and thereby rendered it or its hydrochloride alcohol-insoluble. Partial neutralization of the acidic solution caused the precipitation of the depressor along with salts. Ether extraction removed it from the latter and yielded a light yellow nitrogenous material.

The effect of the depressor material in causing a contraction of duodenal muscle suggests that the material could act directly on the blood vessel wall. It is of interest to note that a decreased stimulation of the muscle strip resulted on repeated application of the depressor. The sustained elevation in blood pressure which occurred when the phosphatide fraction 70-f was given intraperitoneally may have resulted because of stimulation of intestinal contraction.

Because of the vasodepressor effect of phosphatide fraction B(F-2), Emulsion 35¹ is not considered suitable for clinical use, though it is adequate for experimental studies in the dog, rat, or rabbit. Although phosphatide fractions low in depressor activity could be prepared, these were either poor emulsifying agents or, if they were suitable as an emulsifying agent, they underwent changes on autoclaving that gave rise to toxic effects. Fraction C(F-1) (Table III) is a good example of a phosphatide fraction with no depressor activity, good emulsifying properties, but one which is unsatisfactory following autoclaving. In general it appears that the more a phosphatide preparation is purified,

the less stable it becomes to autoclaving. Fraction B(F-2) still remains the most suitable of the soybean phosphatide fractions for stabilizing fat emulsions for intravenous use. Only approximately half of fraction B(F-2) possesses good emulsifying properties, but the remainder of the preparation appears to provide stability to the fraction during autoclaving.

The question arises as to whether fraction B(F-2) can be used in a concentration of less than 3 per cent (as it is used in Emulsion 35) and stabilize an emulsion, yet not be present in such an amount that a depressor effect will be obtained. Emulsions containing 15 per cent fat, identical in composition to Emulsion 35 except that fraction B(F-2) has been reduced from 3 per cent to 0.5 per cent, have been prepared, and they do not affect the blood pressure of the cat. However, stability on standing and when added to plasma is not as great as desired. Further studies utilizing a costabilizer in addition to 0.5 per cent of fraction B(F-2) are reported in the following paper.

SUMMARY

1. A soybean phosphatide preparation, B(F-2), prepared for use as a stabilizer for fat emulsions for intravenous nutrition has been found to possess vasodepressor activity in man and the cat, little such activity in the dog, and none in the rat or rabbit.

2. The depressor material is not present in phosphatide fractions freshly prepared but develops, on standing, on contact with air. It does not appear to be acetylcholine, choline, ethanolamine, or any simple derivatives of these compounds. The depressor material has been concentrated considerably.

3. Fractions of phosphatide low in depressor activity have been prepared, but are either undesirable emulsifying agents or are adversely affected by autoclaving.

We wish to express appreciation to the following companies which have supplied us generously with various materials used in this research: The Upjohn Company, Kalamazoo, Mich.; Merck and Company, Inc., Rahway, N. J.; Gaines Division of General Foods Corporation, Holoken, N. J.; Wilson Laboratories, Chicago, Ill.; Sheffield Farms Co., Inc., New York, N. Y.; Corn Industries Research Foundation, New York, N. Y.; and Anheuser-Busch Co., St. Louis, Mo.

REFERENCES

1. Geyer, R. P., Mann, G. V., Young, J., Kinney, T. D., and Stare, F. J.: Parenteral Nutrition. V. Studies on Soybean Phosphatides as Emulsifiers for Intravenous Fat Emulsions, *J. LAB. & CLIN. MED.* 33: 163, 1948.
2. Mann, G. V., Geyer, R. P., Watkin, D. M., Smythe, R. L., Dju, Dsai-chwen, Zamcheck, N., and Stare, F. J.: Parenteral Nutrition. VII. Metabolic Studies on Puppies Infused with Fat Emulsions, *J. LAB. & CLIN. MED.* 33: 1503, 1948.
3. Geyer, R. P., Chipman, J., and Stare, F. J.: *In Vivo* Oxidation of Emulsified Radioactive Triheaurin Administered Intravenously, *J. Biol. Chem.* 176: 1469, 1948.
4. Lerner, S. R., Chaikoff, I. L., Entenman, C., and Dauben, W. G.: Oxidation of Parenterally Administered C-14-Labeled Tripalmitin Emulsions, *Science* 109: 13, 1949.
5. Collins, H. S., Kraft, L. M., Kinney, T. D., Davidson, C. S., Young, J., and Stare, F. J.: Parenteral Nutrition. III. Studies on the Tolerance of Dogs to Intravenous Administration of Fat Emulsions, *J. LAB. & CLIN. MED.* 33: 143, 1948.
6. Geyer, R. P., Mann, G. V., and Stare, F. J.: Parenteral Nutrition. IV. Improved Techniques for the Preparation of Fat Emulsions for Intravenous Nutrition, *J. LAB. & CLIN. MED.* 33: 153, 1948.
7. Engel, R. W.: Modified Methods for the Chemical and Biological Determination of Choline, *J. Biol. Chem.* 144: 701, 1942.
8. Woolley, D. W.: Isolation and Partial Determination of Structure of Soy Bean Lipositol, a New Inositol Containing Phospholipid, *J. Biol. Chem.* 147: 581, 1943.

The plasma lecithin:cholesterol acyltransferase reaction

JOHN A. GLOMSET

Department of Medicine and Regional Primate Research Center, University of Washington, Seattle, Washington 98105

ABSTRACT Evidence for the existence of a plasma lecithin:cholesterol acyltransferase is reviewed with emphasis not only on the lipid reactants, but also on the lipoprotein "substrates" and "products." The cholesteryl esters of all major lipoprotein classes become labeled when plasma is incubated with cholesterol- ^{14}C . However, the smaller, lecithin-rich high density lipoproteins appear to be preferred substrates. Most studies of factors that influence the acyltransferase reaction have not adequately distinguished between effects on the enzyme and effects on the lipoprotein substrates. However, the fact that cholesterol esterification is diminished in plasma from eviscerated animals or from patients with reduced liver function suggests that the liver may regulate both the level of the enzyme and that of the substrates. Several indications exist that the acyltransferase reaction is the major source of plasma esterified cholesterol in man. Furthermore, the reaction may have a broader, extracellular function. One possibility is that it plays a role in the transport of cholesterol from peripheral tissues to the liver.

KEY WORDS acyltransferase · plasma · lecithin · cholesterol · cholesteryl ester · lysolecithin · lipoproteins · cholesterol transport · peripheral tissues · liver

A NET ESTERIFICATION of cholesterol occurs when plasma, serum, or whole blood is incubated at 37°C . The enzyme that catalyzes the reaction is present in higher concentrations in plasma than in other tissues, and mainly transfers fatty acids from the 2-position of lecithin to cholesterol. For this reason it can be regarded as a plasma lecithin:cholesterol acyltransferase, although

the character of the reaction may depend as much on the nature of the lipoprotein substrates as on the specificity of the enzyme.

The acyltransferase reaction is of interest for several reasons. For example, it appears to be a physiologically important source of plasma cholesteryl esters; an inborn error of metabolism in which the reaction does not occur has recently been discovered; and the reaction shows promise as a tool for studying lipoprotein structure. The purpose of the present review is to discuss the available evidence concerning the mechanism of the reaction, the properties of the enzyme, and the factors and conditions that influence the reaction. In addition, the possible physiological significance of the reaction will be considered.

MECHANISM OF THE REACTION

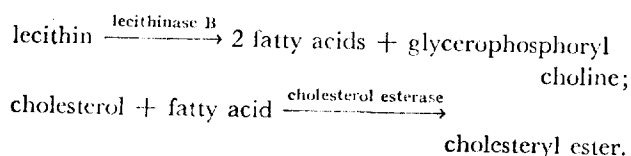
Lipid Reactants

The plasma cholesterol esterification reaction was first described by Sperry (1). He found that maximal esterification of the cholesterol of human plasma occurred at about pH 8, and that the change in cholesterol could be prevented by prior heating of the plasma at $50-60^{\circ}\text{C}$. At first he believed that the reaction was catalyzed by a plasma cholesterol esterase, and suggested that the necessary fatty acids might be derived from plasma phospholipids by the action of a lipase. Later, however, Sperry and Stoyanoff postulated the existence of a special cholesterol-esterifying enzyme to explain their observations (2-4) of the divergent effects of bile salts on the cholesteryl esters of dog, human, and monkey sera. At concentrations of $4-6\text{ }\mu\text{moles/ml}$, bile salts inhibited cholesterol esterification in the sera of all three species, but at higher concentrations they promoted the hydroly-

Abbreviations: HDL, high density lipoproteins; LDL, low density lipoproteins; VLDL, very low density lipoproteins; DEAE-cellulose, diethylaminoethyl cellulose.

sis of cholesteryl esters in dog serum alone. Because of these findings, it was concluded that dog serum contains separate cholesterol-esterifying and cholesteryl ester-hydrolyzing enzymes, whereas human and monkey sera contain only the former.

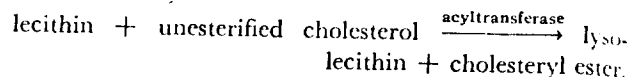
Sperry's observation of the increase in esterified cholesterol during the incubation of serum or plasma was soon confirmed (5, 6), as was his finding (4) that the change could not be accounted for by bacterial contamination (7). Furthermore, Le Breton and Pantaléon (7) obtained evidence that plasma phospholipids indeed might be the source of fatty acids in the esterification reaction. They showed that acetone-precipitable lipid decreased during the incubation of serum, that this decrease and the decrease in unesterified cholesterol could be prevented by adding phlorizin or monobromoacetate, and that adding ovelcithin increased the amount of cholesterol esterified. Because serum incubated for 72 hr did not hemolyze red cells, and because they could not detect acetone-precipitable lipid in some samples of incubated serum, they concluded that the decrease in "phosphoaminolipid" was caused by a lecithinase B. Finally, they proposed a mechanism for the plasma cholesterol esterification reaction that involved the coupled action of the lecithinase B and a plasma cholesterol esterase:



Later, their observation concerning the effect of added lecithin was confirmed by Wagner (8-10) and Murphy (11); and Etienne and Polonovski (12-14) directly demonstrated a decrease in lecithin and an increase in glycerophosphoryl choline in extracts of incubated serum.

During their experiments, Etienne and Polonovski (12-14) also found indications that the changes in plasma phospholipid might be more complicated than originally envisaged by Le Breton and Pantaléon. They showed that lysolecithin transiently increased and that ultimately free choline increased when human serum was incubated for 72 hr. These findings are compatible with the possibility that more than one enzyme may be involved in the breakdown of phospholipid during prolonged incubations of plasma. Moreover, my colleagues and I (15, 16) subsequently obtained results which led us to propose that the initial breakdown of lecithin is not caused by a hydrolase, but by an acyltransferase. We observed (15) that labeled cholesteryl esters were formed in plasma incubated with linoleoyl- ^{14}C lecithin, but not in plasma incubated with linoleate- ^{14}C complexed to albumin. We showed (16) that the relative amount of arachidonic acid released from rat plasma lecithin by

Crotalus adamanteus venom was similar to that which became esterified to cholesterol during the incubation of rat plasma. Finally, we noted (16) that, when human plasma was incubated, the molar change in lecithin was similar to that in unesterified cholesterol. On the basis of these findings we postulated the following:



Furthermore, we suggested that the enzyme mainly transfers fatty acids from the 2-position of lecithin.

Although this reaction formulation subsequently received support from Shah, Lossow, and Chaikoff (17), data exist that do not completely fit it. 10% or more of the fatty acids transferred to cholesterol are saturated (16), a much higher percentage than is released from lecithin by snake venom. Therefore, saturated fatty acids (mostly palmitic acid) may largely be derived from a source other than the 2-position. Plasma triglyceride might be one such source, since cholesteryl palmitate- ^{14}C is formed when plasma is incubated with tripalmitin- ^{14}C (15, 17). Moreover, the transfer from triglyceride to cholesterol may proceed via lecithin, since considerable labeled phosphatide is also formed when plasma is incubated with emulsified tripalmitin- ^{14}C (17). The 1-position of mono- or diacyl glycerophosphoryl choline also might be a source of saturated fatty acid, although no evidence of this exists as yet.

A second observation that does not fit our reaction formulation is that only about one-half the predicted amount of lysolecithin can be demonstrated in human plasma after a 24 hr incubation (18). However, if lysolecithin can function as a donor of saturated fatty acids, as suggested above, or if one or more lysolecithin hydrolases are present in plasma (19), this discrepancy might be explained.

A third objection to our concept is provided by the results of Goodman (20) and of Portman and Sugano (21). Goodman noted that less cholesteryl arachidonate is formed during the incubation of human plasma than would be expected from the composition of the plasma lecithin. This has been confirmed, and contrasts with the good agreement existing with respect to linoleate.¹

¹ Glomset, J., W. King, and Y. Akanuma. Unpublished experiments. We incubated LDL-free human plasma (22) with cholesterol- ^{14}C and compared the pattern of labeled cholesteryl esters formed during a 6 hr incubation with the composition of the 2-fatty acids of lecithin extracted from the unincubated lipoproteins. The cholesteryl esters were separated on thin-layer plates of AgNO_3 -treated silica gel (23). The 2-fatty acids were prepared by treatment of the chromatographically purified lecithin (22) with *Crotalus adamanteus* venom (24), and were subsequently analyzed by gas-liquid chromatography. The relative distribution of radioactivity in the cholesteryl esters was: 12.3% for the saturated esters, and 20.6, 56.6, and 10.5% for the mono-, di-, and tetra-unsaturated esters, respectively. The corresponding figures for the 2-fatty acids were 3.5, 14.5, 56.5, and 25.4%.

Therefore, Goodman's observation suggests that the acyltransferase of human plasma may be able to distinguish between different fatty acids in the 2-position of lecithin. The results of Portman and Sugano (21) also support this possibility. They incubated mixtures of fresh human plasma with heat-inactivated rat plasma labeled with cholesterol- ^{14}C , and fresh rat plasma with heat-inactivated human plasma labeled with cholesterol- ^{14}C and studied the composition of the resulting cholesteryl- ^{14}C esters. In both kinds of experiment they found that the newly formed esters more nearly resembled the preexisting cholesteryl esters of the fresh plasma than those of the unincubated mixture. They concluded that the pattern of cholesteryl esters formed during the incubation was related to the specificity of the enzyme (in the fresh plasma) rather than the composition of the fatty acid source (the lipoproteins of the mixed heated and fresh plasmas). This conclusion appears to be compatible with Goodman's results, because of the implication that the cholesterol-esterifying enzymes of human and rat plasma preferentially transfer linoleate and arachidonate, respectively.

Role of Lipoproteins

Another interpretation of the data of Goodman and of Portman and Sugano is that the specificity of the acyltransferase depends primarily on lipoprotein structure. It is not certain that linoleoyl lecithin and arachidonoyl lecithin occupy similar positions in human plasma lipoproteins. If they do not, they may differ in accessibility to the enzyme, and this, rather than fatty acid specificity per se, could explain Goodman's results. Similarly, the results of Portman and Sugano are compatible with the possibility that the enzymes of human and rat plasma selectively react not with specific fatty acids, but with homologous lipoproteins. Thus, their incubation mixtures contained substrate lipoproteins from both species, and the rat and human lipoproteins differed in relative content of linoleic and arachidonic acid. Therefore, preferential formation of homologous enzyme-substrate complexes could have led to the transfer of different amounts of linoleate and arachidonate. Fortunately, enzyme preparations can now be obtained that contain little lipoprotein (see later discussion). Use of these enzyme preparations instead of whole, fresh plasma should help to determine whether the enzymes of rat and human plasma preferentially react with specific fatty acids or with specific lipoproteins.

That human plasma acyltransferase can react preferentially with specific lipoproteins is suggested by the following. After a 1 hr incubation of human plasma with cholesterol- ^{14}C , the specific activity of HDL cholesteryl esters greatly exceeded that of VLDL or LDL cholesteryl

esters.² Furthermore, when human or baboon plasma was incubated with cholesterol- ^{14}C , the specific activity of the esterified cholesterol also varied among HDL subfractions (22). These differences in cholesteryl ester specific activity probably reflect differences in the enzyme's rate of reaction with different lipoprotein substrates. In fact, calculations of the average rate of cholesterol esterification indicated that four times as much HDL cholesterol was esterified as LDL cholesterol (22). Moreover, the difference may have been even greater, since the calculations did not take into account the transfer of newly formed cholesteryl esters from HDL to LDL. Thus, these studies indicate that the acyltransferase readily differentiates between different lipoproteins.

Lipoprotein structure may influence not only the specificity and rate of the reaction, but also its direction. A significant proportion of the lysolecithin formed by the acyltransferase reaction apparently is not retained by the parent lipoproteins, but rather becomes bound to albumin (18, 25). This not only causes a net loss of phospholipid from the lipoproteins, but could be one reason why esterified cholesterol and lysolecithin are formed at the expense of lecithin. If lysolecithin is not retained by the part of the lipoprotein surface that reacts with the enzyme, reverse transacylation from esterified cholesterol to lysolecithin might be greatly diminished. If this is true, cholesterol esterification might occur less readily in the absence of albumin. This possibility should be explored when albumin-free preparations of plasma acyltransferase become available.

Implicit in the above discussion has been the concept that "substrate" and "product" lipoproteins exist. If the acyltransferase reaction is a physiologically important source of plasma cholesteryl esters (see later discussion), then recently secreted lipoproteins that have not reacted with the enzyme might contain relatively large amounts of lecithin and unesterified cholesterol, whereas lipoproteins that have circulated in the plasma long enough to react maximally with the enzyme might contain less lecithin and more esterified cholesterol. My colleagues and I (22) have reported evidence that lipoproteins of these types are present in plasma. We filtered HDL from human and baboon plasma through columns of Sephadex G-200 and measured the ratios of

² Akanuma, Y., and J. Glomset. Results to be submitted. After a 1 hr incubation of whole human plasma with cholesterol- ^{14}C , VLDL, LDL, and HDL were prepared by ultracentrifugation and chromatography on hydroxylapatite. Lipids were extracted and cholesteryl esters were isolated by thin-layer chromatography (22). In three experiments mean cholesteryl ester specific activities relative to HDL esterified cholesterol were: HDL cholesteryl esters, 1.00; VLDL cholesteryl esters, 0.27; and LDL cholesteryl esters, 0.13.

lecithin to sphingomyelin and of esterified to unesterified cholesterol in the effluent. We also incubated whole blood with labeled cholesterol, filtered the HDL through Sephadex, and measured the specific activity of the esterified cholesterol in the effluent. We found that the HDL subfractions that emerged initially had lower ratios of lecithin to sphingomyelin than those that emerged later. Furthermore, the specific activity of the esterified cholesterol in the initial subfractions was lower than in the later subfractions. On the basis of these results we tentatively identified the initial and final subfractions of the HDL peak as "products" and "substrates" of the acyltransferase reaction, respectively.

The highly provisional nature of this identification can be seen from the following. First, the ratio of esterified to unesterified cholesterol in the initial and final subfractions did not appear to fit our concept, and we had to assume that secondary changes in lipoprotein cholesterol occur in the circulation. Secondly, we assumed that lecithin that has been degraded by the acyltransferase is not replenished, and that the sphingomyelin content does not change while lipoproteins remain in the circulation. Both of these assumptions remain to be verified. Furthermore, both should be considered in the light of the physical exchange known to occur among the plasma lipids. Newly formed cholesteryl esters transfer from HDL to LDL and VLDL (26, 27); unesterified cholesterol probably transfers to HDL from both cells and lipoproteins after HDL cholesteryl esters have been formed by the acyltransferase reaction (11, 22, 28); phospholipids can exchange among lipoproteins (29, 30); and, as already mentioned, much of the lysolecithin formed by the acyltransferase reaction appears to move away from lipoproteins onto albumin (18, 25).

Despite these problems of interpretation, we are continuing our efforts to identify and isolate "substrate" and "product" lipoproteins and are presently focusing our attention on the LDL. Although the acyltransferase appears to react preferentially with HDL, it also reacts with LDL. Therefore, "substrate" and "product" LDL should also exist.

Closely related to the problem of isolating and identifying "substrate" and "product" lipoproteins is the question of the effect of the acyltransferase reaction on the physical properties of lipoproteins. Although this question still has not received much attention, one of the first studies was performed several years ago by Tayeau and Nivet (31, 32). They found that some inhibitors of the acyltransferase reaction prevented or partially prevented the increase in electrophoretic mobility of α - and β -lipoproteins which is well known to occur during incubation or storage (33, 34). On the basis of these results, they concluded that the change in mobility might be caused by the cholesterol esterification reaction. A second study has

already been mentioned, i.e., our study of HDL subfractions (22). We interpreted our results as evidence that the acyltransferase reaction causes aggregation of smaller "substrate" HDL into larger "product" HDL. Both studies emphasize the possibility that the acyltransferase reaction may influence the physical properties of lipoproteins. However, in both cases the conclusions should be regarded as tentative until the existence of experimental artifacts has been ruled out more thoroughly. For example, it is possible that the change in electrophoretic mobility noted by Tayeau and Nivet was caused by the release of fatty acids during the incubation and the subsequent binding of these fatty acids to lipoproteins.

Fortunately, it may soon be possible to test the validity not only of the above concepts, but also of the basic idea of the existence of "substrate" and "product" lipoproteins. Norum and Gjone (35-37) have recently described a new inborn error of metabolism characterized by the apparent absence of acyltransferase activity and by the near absence of cholesteryl esters from plasma. If, indeed, the primary defect in this disease is the absence of plasma lecithin:cholesterol acyltransferase activity and not some related, but independent disorder of lipoprotein structure, the lipoproteins in the blood of these patients should closely resemble the "substrates" postulated to be present in the plasma of normal individuals. One observation, consistent with this possibility, has already been made by Norum and Gjone, i.e., that the ratios of lecithin to sphingomyelin and unesterified to esterified cholesterol in the plasma of these patients are considerably higher than normal.

PROPERTIES OF LECITHIN: CHOLESTEROL ACYLTRANSFERASE

Distribution

Kaplan and I (38) studied the distribution of lecithin:cholesterol acyltransferase-like activity in rat tissues. We used an assay system that consisted of heat-inactivated plasma and an albumin-stabilized emulsion of cholesterol- ^3H , and measured the esterifying activity of tissue homogenates as compared to that of plasma. To reduce contamination of the tissues by blood, we perfused the animals with saline before removing the various tissues, and to compensate for possible extraneous tissue effects, we incubated known amounts of acyltransferase activity with tissue homogenate controls. We found that the concentration of acyltransferase-like activity in blood plasma was several-fold higher than that in any of the tissues. Moreover, the true plasma:tissue ratios may have been even higher because the tissues were contaminated with extracellular fluid. The low level of enzyme activity that we found in the liver is of interest (see later discus-

sion), since this organ possibly may be the source of the plasma enzyme. If the liver does secrete the enzyme into the plasma, it probably does not store the enzyme in active form. The low level of activity in the liver is also of interest in view of the results of Akiyama, Minari, and Sakagami (39), who found that the supernatant fraction of rat liver homogenates contained cholesterol-esterifying activity similar in several respects to the acyltransferase activity of rat plasma. Akiyama et al. (39) did not measure the relative activity of the liver acyltransferase compared to that of plasma. Therefore, the presence of enzyme in their liver homogenates may simply have been a reflection of contamination with extracellular fluid. On the other hand, the pH optimum of the liver supernatant enzyme was 6.5 as opposed to 7.3, the optimum they found for rat plasma. Consequently, a specific liver enzyme may exist. This possibility should be explored, and one of the first experiments performed should be to redetermine, with a common substrate, the pH optima of the rat liver and plasma enzymes.

Purification

Because the plasma acyltransferase has been only partially purified, comparatively little is known about its physical or chemical properties. However, it is more strongly adsorbed to DEAE-cellulose than most plasma proteins are (40). Furthermore, the cholesterol-esterifying activity of human plasma migrates as an α_1 -globulin on zone electrophoresis (the enzyme activity has a somewhat slower mobility than that of α_1 -lipoprotein cholesterol), and it emerges from Sephadex G-200 columns in the same position as α_1 -lipoprotein (40, 22). The last-named observations suggest that the enzyme is either very similar to HDL or is complexed to HDL. That an HDL-enzyme complex can exist is further supported by the results of Lossow, Shah, and Chaikoff (41), who found that significant enzyme activity accompanied the HDL upon ultracentrifugal flotation in a solution of sucrose in D_2O . However, the enzyme can be separated from HDL by centrifugation in KBr solution that has a density of 1.21 g/ml (40).

A method for partial purification of the enzyme that has been suggested (40) is based on a combination of chromatography on DEAE-cellulose and hydroxylapatite, precipitation with ammonium sulfate, and separation of the enzyme from HDL by ultracentrifugation. This method yielded an enzyme preparation that contained only about 20% of the activity of the original plasma, and was only about 30-fold purified. At this stage the principal contaminant appeared to be albumin, as judged by electrophoresis and gel filtration. Clearly, a much more highly purified preparation of the enzyme would be desirable.

FACTORS THAT INFLUENCE THE ACYLTRANSFERASE REACTION

Methods of Assay

In most studies of the plasma acyltransferase reaction, the esterification of cholesterol has been measured by one of three methods: (a) Plasma or serum is incubated for 1-3 days at 37°C, and the change in unesterified cholesterol is determined. (b) Plasma is incubated for 1-3 hr at 37°C, and the initial rate of change in unesterified cholesterol is determined. (c) A relatively small amount of fresh plasma is incubated for 1-6 hr at 37°C with a substrate composed of heat-inactivated plasma and labeled, unesterified cholesterol added as an albumin-stabilized emulsion (37) or coated onto Celite particles (21). Unfortunately, none of these methods are ideal and therefore the results discussed below must be considered with caution. The first method measures essentially the extent of esterification, not the rate. The amount of substrate available (especially lipoprotein lecithin), particularly in the 3-day incubations, is more critical than the level of enzyme activity. Furthermore, when a relatively large amount of unesterified cholesterol is present, and the percentage of change rather than the absolute change in unesterified cholesterol is given, a mistaken impression even of the extent of the reaction can result. The second method depends on the level of enzyme activity and also on the nature of the endogenous, substrate lipoproteins. This is an advantage if the method is to be used to estimate the rate of esterification of plasma cholesterol *in vivo*, but not if the method is to be used to assay enzyme activity *per se*. In the third method the formation of labeled cholesteryl esters is measured. Two fundamental assumptions are made, i.e., that the labeled, unesterified cholesterol in the substrate mixture has equilibrated completely with the unlabeled, unesterified cholesterol of the lipoproteins, and that the enzyme reacts equally well with the preheated substrate lipoproteins and with the endogenous lipoproteins present in the fresh plasma itself. In support of these assumptions, Portman and Sugano (21) have reported that the calculated amount of cholesterol esterified is close to that actually obtained during the incubation of fresh plasma. Nevertheless, the third method can not be regarded as completely independent of endogenous substrate, except when essentially lipoprotein-free preparations of the enzyme are assayed. Moreover, adequate standardization of the third method is difficult because of the limited stability of the heated, substrate lipoproteins.

Factors That Affect the Reaction In Vitro

Tables 1 and 2 list the factors reported to affect the esterification of plasma cholesterol *in vitro*. Several deserve special comment. Exogenous lecithin is interesting (Table 1) because its effect of markedly increasing cho-

TABLE 1 STIMULATION OF CHOLESTEROL ESTERIFICATION IN VITRO

Factor	Method of Measurement	Remarks	Reference
1. Lecithin (exogenous)	Net esterification 1-3 days	1 mg/ml of ovolcithin added to sheep or calf serum	7
"	"	Net esterification 1-8 days	8
"	"	Net esterification 1-3 days	9
"	"	"	11
2. Extracts of Group A streptococci	Net esterification 3 days	2.7 mg/ml of synthetic dimyristoyl lecithin gave maximal effect. Use of higher concentrations not reported	42
3. Polyvalent anions	Radioactive cholesterol assay	1 mg/ml of dimyristoyl lecithin stimulated, but dipalmitoyl lecithin and "dipalmitoyl cephalin" did not	40
		Both rate and extent of reaction increased; accompanied by increased opalescence of serum	
		Phosphate, sulfate, citrate, and EDTA all stimulated in concentrations up to 0.2 N	

TABLE 2 INHIBITION OF CHOLESTEROL ESTERIFICATION IN VITRO

Factor	Method of Measurement	Remarks	Reference
1. Heating at 55-60°C	Net esterification 3 days	1-2 hr	1
"	"	Radioactive cholesterol assay	40
2. Hemolyzed blood	Net esterification 3 days	30 min	1
3. Sodium salts of free and conjugated bile acid	Net esterification 1-2 days	Human blood incubated, then serum analyzed. Partial inhibition	2, 3
Sodium taurocholate	Radioactive cholesterol assay	In human and dog serum complete inhibition with 4 mM glyco-, taurodeoxycholate; 6 mM taurocholate, cholate, deoxycholate	40
4. Phlorizin	Net esterification 3 days	Inhibition by 5 mM of the bile salt reversed by dialysis (human serum)	7
5. Monobromoacetate	"	1 g/liter partially inhibited (dog and human serum)	7
6. <i>p</i> -Hydroxymercuribenzoate	Net esterification 1 day	0.2 g/liter completely inhibited (dog and human serum)	16
7. Thymol	Net esterification 3 days	1-2 mM solution completely inhibited. Reversed by 0.01 M mercaptoethanol	7
8. Cholesterol	"	1 g/liter partially inhibited	8
9. Phospholipase A	Incorporation of labeled precursors into cholesterol esters by extracts of rat plasma acetone powders	Exogenous cholesterol dissolved from glaze on side of incubation tube	17
"	Net esterification 1 day	Preincubation of 2 ml of extract with 0.5 mg of <i>Crotalus adamanteus</i> venom at 37°C for 20 hr completely inhibited	43
10. Phospholipase C	"	Fresh human serum incubated with <i>Crotalus adamanteus</i> venom completely inhibited	43
11. Saponin	Net esterification 3 days	Commercial preparation of <i>Clostridium welchii</i> phospholipase C completely inhibited	44
12. Urea	Radioactive cholesterol assay	5 g/liter said to inhibit, but no details given.	40
13. Diisopropyl fluorophosphate	Net esterification 1 day	4 M urea partially inhibited	42
14. Para-oxon	Estimation of change in lysolecithin on chromatograms after a 15 hr incubation	5×10^{-4} M completely inhibited	45
15. Polyvalent cations	Radioactive cholesterol assay	0.1 mM appeared to inhibit completely	40
		Ca ⁺⁺ and Mg ⁺⁺ inhibited increasingly in concentrations up to 0.2 N	

lesterol esterification suggests that the amount of endogenous lecithin in plasma is an important determinant of the reaction.³ Since the rate of the reaction in fresh plasma decreases long before the lipoprotein lecithin is exhausted, the stimulatory effect of exogenous lecithin

³ This is also suggested by the inhibitory effect of lecithinase shown in Table 2.

further suggests that only a limited amount of the lecithin present can serve as a donor of fatty acids. This obviously may have important implications with respect to lipoprotein structure.

The fact that the acyltransferase is inhibited by sulfhydryl-blocking agents, and reversibly inhibited by *p*-hydroxymercuribenzoate is of interest for practical as well

as theoretical reasons. For example, inhibition of the enzyme is frequently useful during the preparative ultracentrifugation of plasma lipoproteins. Also, the reversible inhibition by *p*-hydroxymercuribenzoate suggests that one or more sulfhydryl groups either form a part of the active center of the acyltransferase or are important in maintaining its configuration.

Physiological and Experimental Conditions In Vivo

Relatively few studies have been done on how the pre-existing state of individual subjects or animals influences the rate of cholesterol esterification in their isolated plasma. Moreover, even fewer studies have been reported in which changes in enzyme activity are effectively differentiated from changes in lipoprotein substrates. Wagner and Poindexter (46) studied the effect of age on the extent of esterification during a 3 day incubation. They reported that the ratio of esterified to unesterified cholesterol after the incubation was higher in 17-20-yr old human females than in "middle aged" females. Unfortunately, they did not include detailed information about the latter. Therefore, whether the actual amounts of cholesterol esterified differed significantly is difficult to determine. Later, Gherondache (47), who reported more detailed results from a similar study, found that both the absolute and relative amounts of cholesterol esterified during a 2 day incubation were greater in 20-39-yr olds than in 40-99-yr olds. Consequently, his results substantiate those of Wagner and Poindexter although they do not distinguish between an effect of age on enzyme activity and an effect of age on the lipoprotein substrates. Therefore, further investigation of the effect of ageing is needed.

Monger and Nestel (48) studied the relation between plasma cholesterol level and rate of esterification in a group of individuals with widely different plasma cholesterol concentrations. They employed an assay system comprised of preheated endogenous lipoproteins, labeled cholesterol, and fresh, "active" plasma; and they found evidence of a direct relationship between the level of cholesterol-esterifying activity and the concentration of plasma cholesterol. However, they did not attempt to determine whether the endogenous lipoproteins in the different plasma samples influenced the apparent level of esterifying activity. The problem of interpreting results such as these, that depend both on the level of enzyme activity and on the type and level of lipoprotein substrate present, is illustrated by the apparent discrepancy between the results of Monger and Nestel (48) and those of Gherondache (47). Gherondache found that less esterification occurred during a 2 day incubation in the sera of older than in that of younger females, even though the older females had higher concentrations of cholesterol. This discrepancy might be related to differences in HDL

levels in the two series since HDL is important in the initial phases of the reaction (22). In any case, the value of separate measurements of enzyme activity and lipoprotein levels should be apparent.

A variable relation appears to exist between sex and plasma cholesterol esterification. Aftergood and Alfin-Slater (49) incubated rat sera for 5 hr, and found that significantly more cholesterol became esterified in the sera of mature females than in that of mature males. On the other hand, Gherondache (47), who measured the extent of esterification in the sera of human males and females, reported data that show no significant sex difference. Finally, Sugano, Chinen, and Wada (50) measured the level of esterifying activity in chicken plasma, using cholesterol-¹⁴C and preheated plasma as a substrate, and found that the level was higher in males than in females (Table 4, below).

The most thorough study of the effect of diet reported is that of Sugano and Portman (51). They investigated the effect of essential fatty acid deficiency (Table 3) by comparing rats fed a fat-free diet, or a diet containing hydrogenated coconut oil, with rats fed a corn-oil diet. They found that more cholesterol became esterified during a 5 hr incubation of the plasma of the deficient rats, even though the deficient plasma contained less than half as much esterified and unesterified cholesterol at the start of the incubation. Aftergood and Alfin-Slater (49) subsequently confirmed this finding.

In order to follow up their initial observation, Sugano and Portman (51) compared the levels of esterifying activity in the essential fatty acid-deficient and control rats. They incubated aliquots of plasma from both kinds of rats with preheated, substrate plasma from both, and found that the level of esterifying activity was higher in the deficient rats irrespective of the substrate used. They also reported that the lipoprotein substrates differed in their capacity to react with the enzyme. Finally, they found that the increased esterification *in vitro* was correlated with an increased turnover of lipoprotein cholesterol *in vivo*.

So far, no comparable studies of the effect of diet in other species have been reported. Favarger (52) reported some studies of the effect of oral fat loads in man which led him to conclude that the rate of esterification of cholesterol in the plasma was increased during the absorption of fat. However, he used too few measurements during the first few hours of incubation to yield reliable results for the initial rate of esterification. Therefore, additional studies will be necessary before his conclusions can be accepted.

Several experimental conditions that affect the esterification of cholesterol in plasma are listed in Table 3. Of these, the effect of evisceration deserves particular mention. Brot, Losso, and Chaikoff (54) removed the liver,

TABLE 3 EFFECT OF EXPERIMENTAL CONDITIONS IN VIVO ON THE ESTERIFICATION OF CHOLESTEROL IN VITRO

Factor or Condition	Method of Measurement	Remarks	Reference
1. Essential fatty acid deficiency	Net esterification 5 hr	Increased in rat plasma	51
" " " "	"	" " "	49
" " " "	Esterifying activity on labeled cholesterol + heated substrate	" " "	51
2. Phlorizin	Net esterification 3 days	Decreased in plasma of dogs injected with phlorizin during previous 2 days	7
3. Ethionine	Esterification of labeled cholesterol	Decreased in rat plasma 1 day after injection of 200 mg	53
4. Evisceration	" " "	Decreased in rat plasma 11 hr after operation	54
5. Ligation of bile duct	Net esterification	Increased in dog plasma 1 wk after ligation; decreased after 3-4 wk	55

gastrointestinal tract, pancreas, and spleen from rats; they obtained blood from the animals 11 hr after the final stage of the operation, and studied the esterification of cholesterol- ^{14}C by the isolated plasma. They found that the plasma from the eviscerated rats esterified less labeled cholesterol, and that this decrease in esterifying activity could not be ascribed to the presence of inhibitors or to the absence of activators. They concluded that their results were most probably due to a diminution in enzyme activity, and suggested that this diminution had been caused by the hepatectomy in view of the known effect of this procedure (56) on the level of cholesteryl esters in plasma. In other words, they inferred that the liver is the source of plasma lecithin:cholesterol acyltransferase. This interesting possibility obviously needs to be substantiated by direct experimentation. For example, the plasma of partially eviscerated animals could be compared with that of animals that have also been hepatectomized, or perfused livers might be used to obtain direct evidence of hepatic synthesis and secretion of the enzyme. A second implication of the results of Brot et al. (54) which should also be explored further is that the acyltransferase of rat plasma is either unstable per se or is rapidly degraded by mechanisms that do not involve the liver, gut, pancreas, or spleen.

Effect of Pathological Conditions

The possibility that the liver plays a role in the synthesis and secretion of plasma lecithin:cholesterol acyltransferase is suggested not only by the experiments of Brot et al. (54) and those of Friedman and Byers (56), but also by the changes in plasma cholesterol in patients with liver disease. In disorders that obstruct the flow of bile, the concentrations of unesterified cholesterol and lecithin in the plasma are increased (57), and the relative, but not necessarily the total, concentration of esterified cholesterol is decreased. In advanced liver failure, the concentrations of all the plasma lipids decrease, especially that of esterified cholesterol. Therefore, plasma acyltransferase activity conceivably might be reduced in either or both of

these conditions, and evidence with respect to both possibilities has been reported.

The possible course of events in biliary obstruction has been indicated by the experiments of Castro Mendoza and Jimenez Diaz (55). They ligated the bile ducts of dogs and studied the acute and chronic effects of this procedure on the plasma cholesterol esterification reaction. 1 wk after the operation the ratio of unesterified to esterified cholesterol had increased, and the absolute concentrations of both had increased. Furthermore, when the plasma of these dogs was incubated for 24 hr, more cholesterol became esterified. However, when they repeated their measurements in two of the dogs after 3 and 4 wk, respectively, the concentration of esterified cholesterol had decreased, and less cholesterol became esterified in vitro. Castro Mendoza and Jimenez Diaz (55) also thought they could discern a similar trend in humans with obstructive jaundice. In most of their patients who had normal or high absolute concentrations of plasma esterified cholesterol, a normal or greater than normal amount of cholesterol became esterified in vitro. On the other hand, when the plasma of two patients with low concentrations of esterified cholesterol was incubated for 24 hr, less esterification occurred. Furthermore, in patients with kala-azar, who had low serum cholesteryl ester concentrations, little cholesterol became esterified in vitro. Because kala-azar often affects the liver, both findings suggest a correlation between the acyltransferase reaction, serum cholesteryl ester concentrations, and liver function.

Turner and his colleagues (58, 59) also studied patients with a number of liver diseases. In general their results in patients with obstructive jaundice appear to agree with those of Castro Mendoza and Jimenez Diaz. However, they expressed their results in terms of the percentage decrease in serum unesterified cholesterol during a 24 hr incubation, and did not include the actual values for cholesterol. Therefore, their results are not strictly comparable. In patients with acute hepatitis they found that the percentage decrease in unesterified cholesterol was

always low during the 1st wk of the illness, whereas esterification increased as the patients improved. Furthermore, they found that esterification was decreased in the sera of many patients with impaired liver function caused by chronic hepatitis, cirrhosis, metastasis, or Hodgkin's disease. Again, however, they expressed their results on the basis of the percentage decrease in unesterified cholesterol during a 24 hr incubation. Since they defined one "enzyme unit" as that "amount of activity capable of producing a 1% decrease in the original level of free cholesterol," the absolute amounts of cholesterol esterified may have been normal or even high in some of their patients. The general problem of interpreting results of this type was briefly discussed earlier. In any study of the effect of physiological or pathological conditions on the acyltransferase reaction an attempt should be made to differentiate between alterations in the plasma lipoprotein substrates and alterations in the level of acyltransferase activity. Moreover, in pathological conditions the presence of inhibitors should also be ruled out. The possibility that soluble cholesteryl ester hydrolase activity, released from tissues as a result of cell damage, might be present should be kept in mind. Both pancreas and liver contain enzymes of this type (60, 61) so that in disorders such as acute hepatitis, hydrolase activity that could offset the activity of the esterifying enzyme might be present in the plasma.

Familial Plasma Cholesteryl Ester Deficiency

This newly discovered inborn error of metabolism (35-37) has already been mentioned briefly. The three sisters who have this disease have normal or increased amounts of cholesterol in their plasma, but less than 10% of it is esterified. Furthermore, no plasma acyltransferase activity is demonstrable either with the patients' own plasma or with heated plasma substrate from normal individuals. Radioactive cholesteryl esters appeared in the plasma of two of these patients after they had ingested cholesterol-³H, but only traces appeared after one of the patients was given an intravenous injection of mevalonate-³H. These findings suggest that the primary defect in this disease may be a genetically determined lack of plasma acyltransferase activity. However, the levels of plasma HDL are abnormally low in this disease, so that the primary defect could involve HDL instead, or some other related, but independent defect in the liver. Despite this uncertainty, the clinical findings are particularly provocative. They are: proteinuria, anemia, hyperlipidemia, and corneal arcus. Moreover, histopathological specimens from bone marrow and kidney contain foam cells.⁴ These findings raise interesting questions

about the physiological role of the acyltransferase reaction.

PHYSIOLOGICAL ROLE OF THE ACYLTRANSFERASE REACTION

Formation of Plasma Cholesteryl Esters In Vivo

A number of reasons exist for believing that the acyltransferase reaction is a physiologically important source of plasma cholesteryl esters. These are:

1. In human plasma the rate of cholesterol esterification in vitro agrees well with the calculated rate of esterification in vivo (Table 4). In other species in which comparisons have been made the rates of esterification in vitro are at least half the calculated rates in vivo (Table 4).

2. The pattern of cholesteryl esters formed by the acyltransferase reaction during the incubation of human plasma is very similar to the pattern of cholesteryl esters in fresh plasma (21). No other enzyme has been shown to yield this pattern. Similarities also exist in the rat (15, 28), but in contrast to man (64), the composition of the VLDL cholesteryl esters differs appreciably from that of the LDL and HDL cholesteryl esters, and does not appear to be compatible with the specificity of the acyltransferase (15, 16, 28, 65). This is particularly true for the cholesterol-fed rat, in which the composition of the VLDL cholesteryl esters, like that of liver cholesteryl esters, is characterized by a predominance of palmitic and oleic acids (65). Both this and the results of tracer experiments (66) suggest that all or nearly all of the VLDL cholesteryl esters may be derived from the liver.⁵

3. The relative incorporation of labeled cholesterol into the cholesteryl esters of human plasma HDL, VLDL, and LDL in vivo (20) is similar to that found in vitro.² Also, the incorporation of labeled cholesterol into the cholesteryl esters of HDL subfractions in the baboon is similar in vivo and in vitro (22).

4. Acyltransferase activity and esterified cholesterol are both absent or nearly absent from plasma in familial plasma cholesteryl ester deficiency. This is clearly significant, even though it has not yet been established whether the mechanisms of cholesterol esterification in the liver are normal in this disease.

5. The concentration of lecithin:cholesterol acyltransferase is considerably higher in plasma than in tissues

⁴ It is worth noting that patients with Tangier disease (familial HDL deficiency) have foam cells that contain cholesterol (62).

⁵ Nevertheless, the results of Heimberg, Van Harken, and Brown (67) may also be mentioned. They perfused livers from rats fed low-cholesterol diets, and found that VLDL released into the perfusate contained few if any cholesteryl esters. Since VLDL prepared from the fresh sera of the same rats did contain cholesteryl esters, these in vitro experiments raise the possibility that many of the cholesteryl esters of rat plasma VLDL may be derived from the plasma in vivo.

TABLE 4 RATES OF ESTERIFICATION OF PLASMA CHOLESTEROL IN VIVO AND IN VITRO

Species	Rate		Reference
	In Vivo	In Vitro	
	$\mu\text{mole/ml/hr}$		
Man	0.12*		63
		0.11†	16
		0.12‡	21
		0.06‡	48
Cebus monkey	0.17*	0.24‡	28
Rat	0.16*	0.08‡	28
Chicken			
Male White Leghorn		0.13‡	50
Female White Leghorn		0.08‡	50
Female White Leghorn (laying)		0.04‡	50

* Calculated on basis of data obtained on injection of mevalonic acid- ^3H .

† Difference in unesterified cholesterol in fresh plasma.

‡ Assayed with heated plasma cholesterol- ^{14}C substrate.

(38), which suggests that the enzyme may have a specific function in plasma.

Reasons for believing that the acyltransferase reaction is *not* an important source of plasma cholesteryl esters have also been advanced. In particular, the liver has seemed the most likely source of the cholesteryl esters of plasma lipoproteins. Plasma cholesteryl ester formation is greatly reduced in hepatectomized animals (56); good evidence exists that most of the plasma lipoproteins are synthesized in the liver (68); the liver contains an active cholesterol-esterifying enzyme system (69); and some investigators have reported that liver cholesteryl esters have a higher specific activity than plasma cholesteryl esters after the injection of labeled cholesterol (70).

However, the effect of hepatectomy is no longer relevant in view of the evisceration experiments of Brot and his colleagues (54) discussed earlier. Furthermore, the existence of plasma lipoproteins that are essentially cholesteryl ester-free in the patients with familial plasma cholesteryl ester deficiency indicates that lipoproteins of this type *can* be synthesized and secreted. The most studied liver cholesteryl-esterifying enzyme, which apparently is the most active (69), preferentially forms cholesteryl oleate and palmitate; these esters do not occur in large amounts in plasma. The importance of the second cholesterol-esterifying enzyme in rat liver (39, 71) as a source of plasma cholesteryl esters remains to be established. Finally, with respect to the isotopic evidence of Swell and Law (70) that the specific activities of cholesteryl linoleate and arachidonate were higher in rat liver than in rat plasma after the injection of unesterified cholesterol- ^{14}C , Sugano and Portman (28) performed similar experiments and reached almost exactly opposite conclusions.

The recent experiments of Gidez, Roheim, and Eder (66) demonstrate the difficulty of interpreting results

based on studies of unfractionated plasma and liver. They injected rats with labeled mevaionate and subfractionated the cholesteryl esters of individual plasma lipoproteins and rat liver organelles after approximately 2 and 8 hr. They showed that the cholesteryl esters of the liver microsomes had the highest specific activities at the earlier time period, and that the specific activities of the saturated and monounsaturated esters were several times higher than those of the most active plasma lipoproteins, the VLDL. On the other hand the specific activity of the microsomal tetraenoic cholesteryl esters was only slightly higher than that of the corresponding HDL esters. The authors concluded that liver microsomes might be the main source of the saturated and monounsaturated cholesteryl esters of the VLDL. However, they also concluded that their data were "not inconsistent with an extrahepatic synthesis of cholesteryl arachidonate on the $d > 1.063$ lipoproteins" because so much more cholesteryl arachidonate was present in plasma than in the liver.

Role of Plasma Acyltransferase in Cholesterol Transport

If the acyltransferase is a physiologically important source of plasma cholesteryl esters, an important question remains to be answered, i.e., why should a specific cholesterol-esterifying enzyme exist in the plasma? One highly tentative possibility is that the acyltransferase reaction plays a role in the transport of cholesterol from peripheral tissues to the liver, and therefore, in a sense, in membrane homeostasis. Some mechanism probably exists for the transport of cholesterol from peripheral tissues, because most peripheral cells can synthesize cholesterol (72), whereas none are known that can catabolize or excrete it.⁶ Furthermore, the experiments of Murphy (11) suggest that the acyltransferase may be part of such a mechanism. He incubated erythrocytes in serum that first had been separately incubated at 37°C for 24 hr, and showed that a net transfer of unesterified cholesterol from the erythrocytes to the serum occurred. He also showed that the loss of erythrocyte cholesterol was related to the amount of esterified cholesterol formed in the serum during the preincubation, and concluded that this loss was due to a shift in the equilibrium between erythrocyte and lipoprotein unesterified cholesterol caused by the esterification reaction. If a similar relation exists between lipoproteins and the cells of tissues such as muscle and kidney, the conditions for a cholesterol transport mechanism might be satisfied. The following evidence is compatible with this possibility:

1. Courtice and Morris (73) have shown that both α_1 - and β -lipoproteins are present in the peripheral lymph of the rabbit; and Voigt, Apostolakis, and Beyer (74)

⁶ Cholesterol can, however, be lost from the body through sloughing of the skin and intestinal mucosa.

have shown that human peripheral lymph also contains soluble lipid that is presumably protein-bound.

2. Peripheral lymph of both humans and monkeys contains cholesterol-esterifying activity, as shown by incubation experiments with labeled cholesterol.⁷

3. The unesterified cholesterol of most peripheral tissues appears to be in equilibrium with that of the plasma lipoproteins (75-77). Therefore, the following sequence of events can be postulated. Plasma lipoproteins (particularly HDL) and plasma lecithin:cholesterol acyltransferase enter the interstitial fluid of peripheral tissues along with other plasma proteins such as albumin (78) and 6.6S γ -globulin (79); lipoprotein cholesterol is esterified by the acyltransferase reaction; the "product" lipoproteins subsequently pick up unesterified cholesterol from cell membranes; finally, the lipoproteins reenter the plasma via the lymph and release their excess cholesterol in the liver. This hypothetical concept is illustrated in Fig. 1.

One objection to this mechanism is that erythrocytes are the only cells for which a net transfer has so far been demonstrated *in vitro*, and a net loss of erythrocyte cholesterol has not yet been demonstrated *in vivo*. A loss may be found when better methods for separating young and old erythrocytes become available. However, it is also possible that very little loss normally occurs because of compartmentation within the body. Thus, the cells of the blood and liver can be viewed as occupying one compartment which is partially separated by capillary endothelium from a second compartment which contains cells of other tissues. Blood cells and liver cells can be included in the same compartment for present purposes because of the relatively unrestricted diffusion of plasma proteins between the hepatic intravascular space and the space of Disse (80). On the other hand diffusion between the first and the second compartment is limited, particularly in the case of the larger plasma proteins (81). Both compartments contain plasma lipoproteins and plasma acyltransferase, and in both of them unesterified cholesterol in cell membranes is in equilibrium with the unesterified cholesterol of circulating lipoproteins. The concept, then, is that the deficit in lipoprotein unesterified cholesterol in the first compartment is largely made up by the liver cells, whereas the deficit in lipoprotein cholesterol in the second compartment is made up by peripheral cells be-

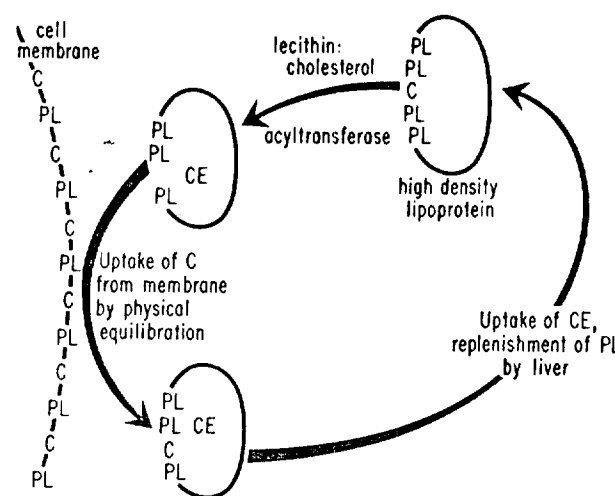


Fig. 1. Postulated mechanism for the transport of cholesterol from membranes of peripheral cells to the liver. Lecithin:cholesterol acyltransferase reacts with circulating lipoproteins to form cholesteryl esters from unesterified cholesterol and lecithin. The lipoproteins subsequently pick up unesterified cholesterol from cell membranes, circulate through the liver, and release esterified cholesterol. C, unesterified cholesterol; CE, cholesteryl ester; PL, phospholipid.

cause the circulating interstitial fluid proteins remain out of contact with the liver for relatively long periods (82).

Other Roles of the Acyltransferase Reaction

Since the acyltransferase reaction is often referred to as the "plasma cholesterol esterification reaction," a role of the reaction in cholesterol transport might seem logical. However, the possibility that esterified cholesterol is only a by-product, and that the principal physiological role of the reaction is connected with the changes in lipoprotein phospholipid, should also be considered. It would be surprising if the conversion of lecithin to lysolecithin and the subsequent loss of lysolecithin from lipoproteins did not have important effects on lipoprotein structure and metabolism. Furthermore, the lysolecithin formed by the reaction is probably metabolized rapidly by cells of the liver and other tissues (83); and studies have only just begun of the physiological role of this lysolecithin. Finally, the fact that patients with familial plasma cholesteryl ester deficiency have anemia and proteinuria introduces the possibility that the acyltransferase reaction may affect the still poorly understood mechanisms that control erythrocyte destruction and the handling of plasma proteins by the kidney.

CONCLUSIONS

The data discussed in this review indicate that the plasma cholesterol esterification reaction is catalyzed by a plasma lecithin:cholesterol acyltransferase, and support the con-

⁷ Glomset, J. Unpublished results. Peripheral lymph was obtained from the leg of a patient with a lymph fistula. An aliquot was incubated with 9 volumes of substrate composed of cholesterol-¹⁴C and heated human plasma (40), and the amount of labeled cholesteryl ester formed was compared with that formed by a similarly incubated aliquot of the patient's plasma. The lymph:plasma ratio of cholesterol esterifying activity was 1:22. A similar experiment was also performed with subcutaneous lymph obtained from the leg of a pigtail monkey. In this experiment, the ratio of lymph enzyme to plasma enzyme activity was 1:10.

clusion that the reaction is a physiologically important source of plasma cholesteryl esters. However, they leave many questions unanswered. One question concerns the role of lipoproteins in the reaction, and the effect of the reaction on lipoproteins. If the interaction between the enzyme and its lipoprotein substrates is relatively specific, the enzyme may be useful in the study of natural and reconstituted lipoproteins. A second question concerns the nature of the factors that coordinate the formation and breakdown of plasma cholesteryl esters with the formation and breakdown of plasma lipoproteins. Considerably more information will be required to explain how a relatively constant ratio of esterified to unesterified cholesterol is maintained in the plasma. Finally, the physiological role of the reaction remains to be clarified. If plasma cholesteryl esters are formed extracellularly, it would be logical to infer that the reaction has a broader, extracellular function. At present little is known about the factors that control the metabolism of plasma lipoproteins once they have entered the circulation, and knowledge of the metabolic interaction between circulating lipoproteins and the lipoproteins of cell membranes is almost as limited. The plasma lecithin:cholesterol acyltransferase reaction could play a role in either or both of these areas.

Warmest thanks are due to Doctors Norum and Gjone for granting me access to their manuscripts.

Our investigations of the acyltransferase reaction have been supported by grants from the Washington State Heart Association and the U. S. Public Health Service, HE-10642.

Manuscript received 4 October 1967.

REFERENCES

- Sperry, W. M. 1935. *J. Biol. Chem.* **111**: 467.
- Sperry, W. M., and V. A. Stoyanoff. 1937. *J. Biol. Chem.* **117**: 525.
- Sperry, W. M., and V. A. Stoyanoff. 1937. *J. Biol. Chem.* **121**: 101.
- Sperry, W. M., and V. A. Stoyanoff. 1938. *J. Biol. Chem.* **126**: 77.
- Klein, W. 1938. *Z. Physiol. Chem.* **254**: 1.
- Pantaléon, J. 1943. *Compt. Rend. Soc. Biol. (Paris)*. **137**: 609.
- Le Breton, E., and J. Pantaléon. 1947. *Arch. Sci. Physiol.* **1**: 199.
- Wagner, A., and L. Rogalski. 1952. *J. Lab. Clin. Med.* **40**: 324.
- Wagner, A. 1955. *Circ. Res.* **3**: 165.
- Wagner, A. 1960. *Quart. Bull. Northwestern Univ. Medical School*. **33**: 282.
- Murphy, J. R. 1962. *J. Lab. Clin. Med.* **60**: 86.
- Etienne, J., and J. Polonovski. 1959. *Bull. Soc. Chim. Biol.* **41**: 805.
- Etienne, J., and J. Polonovski. 1959. *Bull. Soc. Chim. Biol.* **41**: 813.
- Etienne, J., and J. Polonovski. 1960. *Bull. Soc. Chim. Biol.* **42**: 857.
- Glomset, J. A., F. Parker, M. Tjaden, and R. H. Williams. 1962. *Biochim. Biophys. Acta*. **53**: 398.
- Glomset, J. A. 1962. *Biochim. Biophys. Acta*. **65**: 128.
- Shah, S. N., W. J. Lossow, and I. L. Chaikoff. 1964. *Biochim. Biophys. Acta*. **84**: 176.
- Glomset, J. A. 1963. *Biochim. Biophys. Acta*. **70**: 389.
- Dawson, R. M. C. 1956. *Biochem. J.* **64**: 192.
- Goodman, D. S. 1964. *J. Clin. Invest.* **43**: 2026.
- Portman, O. W., and M. Sugano. 1964. *Arch. Biochem. Biophys.* **105**: 532.
- Glomset, J., E. Janssen, R. Kennedy, and J. Dobbins. 1966. *J. Lipid Res.* **7**: 638.
- Goodman, D. S., and T. Shiratori. 1964. *J. Lipid Res.* **5**: 578.
- Saito, K., and D. J. Hanahan. 1962. *Biochemistry*. **1**: 521.
- Switzer, S., and H. A. Eder. 1965. *J. Lipid Res.* **6**: 505.
- Rehnborg, C. S., and A. V. Nichols. 1964. *Biochim. Biophys. Acta*. **84**: 596.
- Nichols, A. V., and L. Smith. 1965. *J. Lipid Res.* **6**: 206.
- Sugano, M., and O. W. Portman. 1964. *Arch. Biochem. Biophys.* **107**: 341.
- Kunkel, H. G., and A. G. Bearn. 1954. *Proc. Soc. Exptl. Biol. Med.* **86**: 887.
- Eder, H. A., J. H. Bragdon, and E. Boyle. 1954. *Circulation*. **10**: 603. (Abstr.)
- Tayeau, F. 1955. *Exposés Ann. Biochem. Med.* **72**: 213.
- Tayeau, F., and R. Nivet. 1956. In *Biochemical Problems of Lipids*. G. Popják and E. Le Breton, editors. Butterworth Scientific Publications, London. 365.
- Armstrong, S. H., Jr., M. J. E. Budka, and K. C. Morrison. 1947. *J. Am. Chem. Soc.* **69**: 416.
- Hoch, H., and A. Chanutin. 1954. *J. Biol. Chem.* **209**: 661.
- Norum, K. R., and E. Gjone. 1967. *Biochim. Biophys. Acta*. In press.
- Gjone, E., and K. R. Norum. 1967. *Acta Med. Scand.* In press.
- Norum, K. R., and E. Gjone. 1967. *Scand. J. Clin. Lab. Invest.* In press.
- Glomset, J. A., and D. M. Kaplan. 1965. *Biochim. Biophys. Acta*. **98**: 41.
- Akiyama, M., O. Minari, and T. Sakagami. 1967. *Biochim. Biophys. Acta*. **137**: 525.
- Glomset, J. A., and J. L. Wright. 1964. *Biochim. Biophys. Acta*. **89**: 266.
- Lossow, W. J., S. N. Shah, and I. L. Chaikoff. 1966. *Biochim. Biophys. Acta*. **116**: 172.
- Rowen, R., and J. Martin. 1963. *Biochim. Biophys. Acta*. **70**: 396.
- Rowen, R. 1964. *Biochim. Biophys. Acta*. **84**: 761.
- Tayeau, F., and R. Nivet. 1955. *Compt. Rend. Acad. Sci.* **240**: 567.
- Vogel, W. C., and E. L. Bierman. 1967. *J. Lipid Res.* **8**: 46.
- Wagner, A., and C. A. Poindexter. 1952. *J. Lab. Clin. Med.* **40**: 321.
- Gherondache, C. N. 1963. *J. Clin. Endocrinol. Metab.* **23**: 1024.
- Monger, E. A., and P. J. Nestel. 1967. *Clin. Chim. Acta*. **15**: 269.
- Aftergood, L., and R. B. Alfin-Slater. 1967. *J. Lipid Res.* **8**: 126.
- Sugano, M., I. Chinen, and M. Wada. 1965. *J. Biochem. (Tokyo)*. **58**: 285.
- Sugano, M., and O. W. Portman. 1965. *Arch. Biochem. Biophys.* **109**: 302.
- Favarger, P. 1946. *Arch. Int. Pharmacodyn.* **72**: 1.

53. Lossow, W. J., S. N. Shah, M. Brot, and I. L. Chaikoff. 1963. *Biochim. Biophys. Acta*. 70: 593.
54. Brot, N., W. J. Lossow, and I. L. Chaikoff. 1962. *J. Lipid Res.* 3: 413.
55. Castro Mendoza, H., and C. Jimenez Diaz. 1949. *Bull. Inst. Med. Res. (Madrid)*. 2: 81.
56. Friedman, M., and S. O. Byers. 1955. *J. Clin. Invest.* 34: 1369.
57. Phillips, G. B. 1960. *J. Clin. Invest.* 39: 1639.
58. Turner, K. B., and V. Pratt. 1949. *Proc. Soc. Exptl. Biol. Med.* 71: 633.
59. Turner, K. B., G. H. McCormack, Jr., and A. Richards. 1953. *J. Clin. Invest.* 32: 801.
60. Hernandez, H. H., and I. L. Chaikoff. 1957. *J. Biol. Chem.* 228: 447.
61. Deykin, D., and D. S. Goodman. 1962. *J. Biol. Chem.* 237: 3649.
62. Fredrickson, D. S. 1966. In *The Metabolic Basis of Inherited Disease*. J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson, editors. McGraw-Hill Book Co., New York. 2nd edition. 486-508.
63. Nestel, P. J., and E. A. Monger. 1967. *J. Clin. Invest.* 46: 967.
64. Goodman, D. S., and T. Shiratori. 1964. *J. Lipid Res.* 5: 307.
65. Gidez, L. I., P. S. Roheim, and H. A. Eder. 1965. *J. Lipid Res.* 6: 377.
66. Gidez, L. I., P. S. Roheim, and H. A. Eder. 1967. *J. Lipid Res.* 8: 7.
67. Heimberg, M., D. R. Van Harken, and T. O. Brown. 1967. *Biochim. Biophys. Acta*. 137: 435.
68. Scanu, A. M. 1965. *Advan. Lipid Res.* 3: 63.
69. Goodman, D. S., D. Deykin, and T. Shiratori. 1964. *J. Biol. Chem.* 239: 1335.
70. Swell, L., and M. D. Law. 1966. *Arch. Biochem. Biophys.* 113: 143.
71. Swell, L., and M. D. Law. 1967. *Biochem. Biophys. Res. Commun.* 26: 206.
72. Dietschy, J. M., and M. D. Siperstein. 1967. *J. Lipid Res.* 8: 97.
73. Courtice, F. C., and B. Morris. 1955. *Quart. J. Exptl. Physiol.* 40: 138.
74. Voigt, K. D., M. Apostolakis, and H. K. Beyer. 1967. *Experientia*. 23: 355.
75. Avigan, J., D. Steinberg, and M. Berman. 1962. *J. Lipid Res.* 3: 216.
76. Chobanian, A. V., B. A. Burrows, and W. Hollander. 1962. *J. Clin. Invest.* 41: 1738.
77. Field, H., Jr., L. Swell, P. E. Schools, Jr., and C. R. Treadwell. 1960. *Circulation*. 22: 547.
78. Berson, S. A., R. S. Yalow, S. S. Schreiber, and J. Post. 1953. *J. Clin. Invest.* 32: 746.
79. Solomon, A., T. A. Waldman, and J. L. Fahey. 1963. *J. Lab. Clin. Med.* 62: 1.
80. Brauer, R. W. 1963. *Physiol. Rev.* 43: 115.
81. Barth, W. F., R. D. Wochner, T. A. Waldmann, and J. L. Fahey. 1964. *J. Clin. Invest.* 43: 1036.
82. Areskog, N.-H., G. Arturson, and G. Grotte. 1964. *Acta Physiol. Scand.* 62: 209.
83. Stein, Y., and O. Stein. 1966. *Biochim. Biophys. Acta*. 116: 95.

Joint FAO/WHO Expert Committee on Food Additives.
1964
Specifications for identity and purity of food
additives and their toxicological evaluation:
emulsifiers, stabilizers, bleaching and maturing agents
World Health Organization, Geneva, Seventh Report,
WHO Tech. Rep. No. 281

Proc. Soc. Exp. Biol. Med. 49(1): 71-73, 1942

13467 P

Experimental Atherosclerosis and Soya Lecithin.*

HOMER D. KESTEN AND RUTH SILBOWITZ.

From the Department of Pathology, College of Physicians and Surgeons, Columbia University, New York City.

The well established lipotropic property of lecithin in the prevention of fatty livers and the partial protection apparently afforded by soy bean flour against cholesterol induced atherosclerosis in rabbits,¹ suggested the investigation of the effect of soya lecithin on experimental atherosclerosis. Accordingly 23 young adult chinchilla rabbits were divided into 3 groups. All were fed 150 mg of cholesterol daily in oil added to a basic diet consisting of white flour, alfalfa, linseed meal, carrots and salt mixture.¹ Group A received nothing more. Rabbits of Groups B and C received 5 g and 1 g respectively in the diet daily of crude soya lecithin (approx-

* This work has been aided by a grant from the American Lecithin Company, Inc.

mate composition: lecithin 20%, cephalin 20%, oil 30%, phytosterols 2%, inositol and allied compounds 15%, carbohydrates 10%). The oil content of the several diets was adjusted to equality. The rabbits were killed after 4 months of feeding and the aortas and viscera examined, both grossly and histologically.¹

Table I indicates the findings. Seven of the 8 animals receiving cholesterol alone developed atherosclerosis of the aorta (for the most part of moderate degree). Only 2 of the 7 in Group B that received the addition of 5 g of soya lecithin daily developed lesions (of minimal degree), and 2 of the 8 in Group C that consumed 1 g of lecithin. The average level of blood cholesterol of the rabbits, in Group A, attained 430 mg (± 150) per 100 cc, in Group B (5 g lecithin) 210 mg (± 75), and in Group C (1 g lecithin) 300 mg (± 100). The livers of all the rabbits were normal grossly and histologically.

How much of this protective effect of lecithin on cholesterol deposition is due to choline is problematical. Choline has been found by Steiner,² Baumann and Rusch,³ and Himsworth⁴ to have no effect on the hypercholesterolemia of cholesterol-fed animals. Baumann and Rusch, and Himsworth reported also no effect on cholesterol deposition in the aorta, but Steiner observed a delay in the production of atherosclerosis. A 4th group of rabbits received 195 mg of choline chloride per rabbit per day (equivalent to the choline content of 5 g of crude soya lecithin). The incidence of atherosclerosis was sharply less than in the animals receiving cholesterol alone, but only a little greater in degree than in the groups fed lecithin. However, hypercholesterolemia in this group [340 mg (± 160) per 100 cc] was more severe than in the animals receiving 5 g of lecithin daily. It

TABLE I.
Effect of Soya Lecithin on Cholesterol Sclerosis.

Diet	Rabbits			Degree of sclerosis of aorta	
	No. used	No. sclerotic	Percentage sclerotic	+	++
A. Basic	8	7	88	2	5
B. Lecithin 5 g	7	2	28	2	0
C. " 1 "	8	2	25	1	1
D. Choline	8	3	38	1	2

+ No lesion visible grossly, or doubtful, but one was evident microscopically.
++ Sclerosis evident grossly as one to several atheromatous plaques.

¹ Meeker, D. R., and Kesten, H. D., *Arch. Path.*, 1941, **31**, 147.

² Steiner, A., *Proc. Soc. Exp. Biol. and Med.*, 1931, **38**, 231.

³ Baumann, C. A., and Rusch, H. P., *Proc. Soc. Exp. Biol. and Med.*, 1938, **38**, 647.

⁴ Himsworth, H. P., *Acta Med. Scand. Suppl.*, 1938, **90**, 158.

seems likely that, with this high blood cholesterol, the animals fed choline would shortly have developed more extensive atherosclerosis. Further work is needed to clarify this point.

Summary. The feeding of soya lecithin to rabbits receiving cholesterol restricts hypercholesterolemia and diminishes the incidence of experimental arteriosclerosis.

Incorporation of Dietary Lecithin and Lysolecithin into Lymph Chylomicrons in the Rat*

(Received for publication, May 31, 1967)

ROBERT O. SCOW

From the Laboratory of Nutrition and Endocrinology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014

YECHIZKIEL STEIN

From the Lipid Research Laboratory, Department of Medicine B, Hadassah University Hospital, Jerusalem, Israel

OLGA STEIN

From the Department of Experimental Medicine and Cancer Research, Hebrew University Hadassah Medical School, Jerusalem, Israel

SUMMARY

Incorporation of dietary phospholipids into lymph chylomicrons was studied in rats fed corn oil containing either radioactive lecithin or lysolecithin. The specific activity of lecithin- ^{32}P in chylomicrons relative to that in the oil increased directly with the amount of lecithin fed, whereas the lecithin content of chylomicrons was constant. About 40% of the phospholipid in chylomicrons was derived from the meal when the lecithin content of the meal was 6 to 11%. When 1-palmitoyl-9,10- ^3H -lecithin- ^{32}P was fed, one-fifth of the ^3H recovered in chylomicrons was present in lecithin and the ratio of ^3H : ^{32}P in lecithin in chylomicrons was the same as that in the oil. When 1-palmitoyl-9,10- ^3H , 2-linoleoyl-1- ^{14}C -lecithin- ^{32}P was fed, 5% of the ^{14}C recovered in chylomicrons was found in lecithin, and the ratios of ^{14}C : ^3H and of ^{14}C : ^{32}P in chylomicron lecithin were only 20% of that in the lecithin fed. The ratio of choline- ^3H to ^{32}P in lecithin of chylomicrons collected from rats fed choline-(methyl)- ^3H lecithin- ^{32}P was the same as that in the oil meal. Up to 40% of 1-acyl lysolecithin- ^{32}P fed with corn oil was recovered in chylomicron lecithin in 6 hours. The findings indicate that dietary lecithin is hydrolyzed and absorbed as lysolecithin and subsequently reacylated before being incorporated into lymph chylomicrons.

* This research was supported in part by Research Grant H-5705 from the National Institutes of Health, United States Public Health Service, Bethesda, Maryland.

Absorption of phospholipids from the intestines was studied in rats by Artom and Swanson (1) by measuring the incorporation of ^{32}P from either phospholipids, inorganic phosphate, or glycerol phosphate into phospholipids of the blood. They found that the specific activity of serum phospholipids was much higher in rats fed labeled phospholipids (isolated from liver) than in those fed labeled inorganic phosphate with unlabeled phospholipid. They concluded that some of the phospholipid fed was absorbed as intact molecules. Bloom *et al.* (2) found in rats a higher recovery of palmitic acid ^{14}C in chyle phospholipids when the labeled fatty acid was fed in the form of hepatic phospholipids than in the form of tripalmitin. They interpreted their findings as showing that some of the phospholipid fed escaped hydrolysis and was incorporated intact into chyle phospholipids. Blomstrand (3) corroborated these findings with studies in the rat with doubly labeled phospholipids, labeled either in the glycerol and fatty acid moieties or in the fatty acid and phosphate moieties.

Since lecithin is the predominant phospholipid both in liver (4) and in lymph chylomicrons (5, 6), the above findings would suggest that lecithin is absorbed intact from the intestines. On the other hand, there is evidence that lecithin is readily hydrolyzed to lysolecithin in the intestines (7) and that the small intestines contain a very active lysolecithin-acylating system (8). The present study was undertaken to test the hypothesis that dietary lecithin is hydrolyzed to lysolecithin, absorbed, and reacylated before being incorporated into chylomicrons of intestinal lymph.

EXPERIMENTAL PROCEDURE

Preparation of Labeled Phospholipids—Rat liver lecithin (diacylglycerolphosphoryl choline) labeled with either ^{32}P or palmitate-9,10- ^3H was prepared as previously described (9). Lysolecithin (1-acylglycerolphosphoryl choline) labeled in either the phosphate or fatty acid was prepared from rat liver lecithin with *Crotalus adamanteus* venom according to the method of Long and Penny (10). 1-Palmitoyl-9,10- ^3H , 2-linoleoyl-1- ^{14}C lecithin (1-palmitoyl-9,10- ^3H , 2-linoleoyl-1- ^{14}C glycerolphosphoryl choline) was prepared by acylating 1-palmitoyl-9,10- ^3H -lysolecithin (1-palmitoyl-9,10- ^3H -glycerolphosphoryl choline) with linoleic acid-1- ^{14}C by the method of Robertson and Lands (11). (The above radioactive materials were purchased from the Radiochemical Centre, Amersham, Great Britain.) Soybean lecithin was labeled with either ^{32}P or choline (methyl)- ^3H by the method of Holzl, Chatterjee, and Hochhammer (12). (Choline chloride (methyl)- ^3H was purchased from New England Nuclear Corporation.) Germinating soybeans were incubated with labeled phosphate or choline for 24 hours at 37°. The beans were then minced and homogenized in a Waring Blender with 20 volumes of chloroform-methanol (2:1, v/v). The mixture was filtered and the residue was extracted overnight with 20 volumes of chloroform-methanol at room temperature. The lipid extracts were combined and purified according to the method of Folch, Lees, and Sloane Stanley (13), and the lecithin was separated on a silicic acid column (14).

The purity of each batch of lecithin and lysolecithin was ascertained by thin layer chromatography (15), and those batches used were more than 98% pure. Unlabeled lecithin isolated from rat liver was used as diluent. Lecithin and lysolecithin were dissolved in diethyl ether and mixed with corn oil with a Vortex Junior mixer (Fisher Scientific Company). The ether was then evaporated with a stream of nitrogen or under reduced pressure.

Collection of Lymph—Female rats of the Hebrew University strain fed Purina chow and weighing 100 to 200 g were used in these studies. On the day before the experiment the rats were

anesthetized with ether and a cannula made of PE-50 (polyethylene) tubing (Clay-Adams, Inc.) was inserted into the abdominal portion of the thoracic duct (anterior to the celiac chyl) according to the method of Bollman, Cain, and Chaffee (16). The rats were then placed in individual restraining cages and given 0.85% NaCl solution to drink and nothing to eat.

On the day of the experiment the rats were fed by tube 0.5 or 1.0 ml of corn oil containing labeled phosphochyle was collected in chilled glass tubes for three periods of 1 hour each. The flow rate averaged 1.9 ml per hour. In individual animals the flow ranged from 0.5 to 4.0 ml per hour. Chyle was kept at 2-4° until separated into chylomicron and infranatant fractions. Clots in the chyle were removed by stirring them on a wooden stick.

The chyle collected during each 2-hour period was diluted with cold 0.85% NaCl solution to a final volume of 8 ml. Five milliliters were taken for analyses of whole chyle and 5 ml were added to a nitrate cellulose tube and centrifuged for 1 hour at 4000 rpm (458,000 $\times g$) in a swinging bucket rotor SW-20 (Beckman Spinco model L ultracentrifuge at 2°).

The infranatant fraction was removed at once by draining through a small pinhole made in the lower portion of the nitrate cellulose tube. The first 3 ml collected were used for analyses of the infranatant fraction. The compact chylomicron layer which formed during centrifugation usually stuck to one side of the tube when the infranatant fraction was removed. The portion of the tube to which the cake was attached was cut out and washed with 5 ml of 0.85% NaCl solution at room temperature. If the chylomicron cake did not stick to the wall of the tube, it was collected at the bottom of the tube by draining off all of the infranatant fraction. The chylomicrons were suspended in 0.85% NaCl by gentle aspiration and expression with a glass Pasteur pipette. Three milliliters of the suspension were taken for analyses of the composition of the chylomicrons. The total amount of lipid constituents in chylomicrons, however, was determined by difference between that present in whole chyle and

TABLE I
Lipid and lipid- ^{32}P content of thoracic duct lymph in rats tube-fed corn oil containing different amounts of lecithin labeled with ^{32}P ^a

Rat No.	Lecithin content of oil ^b	Amount of oil fed	Chyle				Chylomicron			
			Total lipids		Phospholipids		Total lipids		Phospholipids	
			mg	mg	mg	% total lipids	mg	mg	% total lipids	% dose
16	0.06	920	340	23.7	7.0	19	318	13.4	4.2	16
17	0.06	460	178	13.8	7.8	19	149	7.4	5.0	15
18	0.4	340	95	8.0	8.5	19			6.2 ^c	
19	0.6	340	106	12.6	11.8	14			5.2 ^c	
20	1.6	340	98	7.1	7.0	9			3.9 ^c	
23	3.5	400	136	15.8	11.6	8	117	8.8	6.1	6
26	6.5	420	130	13.0	10.0	11		6.0	5.5	9
24	6.5	420	297	24.0	8.1	27		15.0	4.1	22
29	7.4 ^d	440	282	17.5	6.3	18		11.6	3.3	15
30	7.4 ^d	440	250	14.8	5.9	18		8.9	3.3	15
31	7.4 ^d	440	267	17.8	6.7	17		11.0	3.6	15
22	7.9	480	225	13.9	6.2	13	200	6.3	4.7	10
25	11.1	455	228	18.8	8.3	13		11.8	4.3	19

^a Lymph was collected for 6 hours after tube feeding.

^b Lecithin- ^{32}P isolated from rat liver was added to the oil fed to all rats except Rats 29, 30, and 31 (see Footnote d).

^c Measured in chylomicrons collected during the first 4 hours after feeding.

^d Lecithin- ^{32}P isolated from soybean and diluted 1:15 with nonlabeled lecithin isolated from rat liver was added to oil.

that present in the infranatant fraction. All aliquots for analyses were taken on the day of the experiment.

Analytical Methods—Lipids in chyle, chylomicrons, infranatant fraction, and corn oil were extracted with chloroform-methanol (2:1) and purified according to the method of Folch *et al.* (13). Lipids were separated on silicic acid columns by the method of Newman, Liu, and Zilversmit (14). Neutral lipids and free fatty acids were eluted with 5% methanol in chloroform; phosphatidyl ethanolamine and serine with 20% methanol in chloroform; lecithin with 42% methanol in chloroform; and lysolecithin and sphingomyelin with methanol alone. The methanol contained 3% water. Thin layer chromatography (15) was used to verify purity of fractions. Lipid fractions were analyzed for total esterified fatty acid content (17) and phosphorus content (18).

Radioactivity was determined in a liquid scintillation spectrometer (Packard Tri-Carb model 4322) with 10 ml of toluene containing 0.4% diphenyloxazole and 0.01% *p*-bis-[2-(5-phenyloxazolyl)]benzene as the scintillating fluid. Fractions scraped from the thin layer chromatographic plates were suspended in the scintillation fluid with Cab-O-Sil (19). The gain and discriminators in each channel were set so that ^{32}P was counted with a relative efficiency of 100% in Channel A, 3% in Channel B, and 30% in Channel C; ^3H was counted with a relative efficiency of 100% in Channel B, 0.2% in Channel C, and was totally discriminated from Channel A; and ^{14}C was counted with a relative efficiency of 100% in Channel C, 40% in Channel B, and was totally discriminated from Channel A. Relative specific activity of ^{32}P in phospholipids of the various lymph fractions was determined by dividing the specific activity (^{32}P :P) in each fraction by the specific activity (^{32}P :P) in the oil meal.

TABLE II

Relative specific activity of phospholipids of thoracic duct lymph of rats tube-fed corn oil containing different amounts of lecithin labeled with ^{32}P ^a

Rat No.	Lecithin content of oil ^b	Relative specific activity of phospholipids in		
		Chyle	Chylomicrons	Infranant
	%	$(^{32}\text{P}:P)/(^{32}\text{P}:P) \text{ in oil}$		
16	0.06	0.0034	0.0058	
17	0.06	0.0028	0.0042	
18	0.4	0.029	0.041 ^c	
19	0.6	0.026	0.029 ^c	
20	1.6	0.103	0.124 ^c	
23	3.5	0.081	0.096	0.052
26	6.5	0.225	0.298	0.099
24	6.5	0.314	0.440	0.156
29	7.4 ^d	0.332	0.370	0.173
30	7.4 ^d	0.385	0.412	0.165
31	7.4 ^d	0.303	0.394	0.127
22	7.9	0.250	0.220	0.054
25	11.1	0.349	0.460	0.205

^a Lymph was collected for 6 hours after tube feeding.

^b Lecithin- ^{32}P isolated from rat liver was added to the oil fed to all rats except Rats 29, 30, and 31 (see Footnote d).

^c Measured in chylomicrons collected for first 4 hours after tube feeding.

^d Lecithin- ^{32}P isolated from soybean and diluted 1:5 with non-labeled lecithin isolated from rat liver was added to the oil.

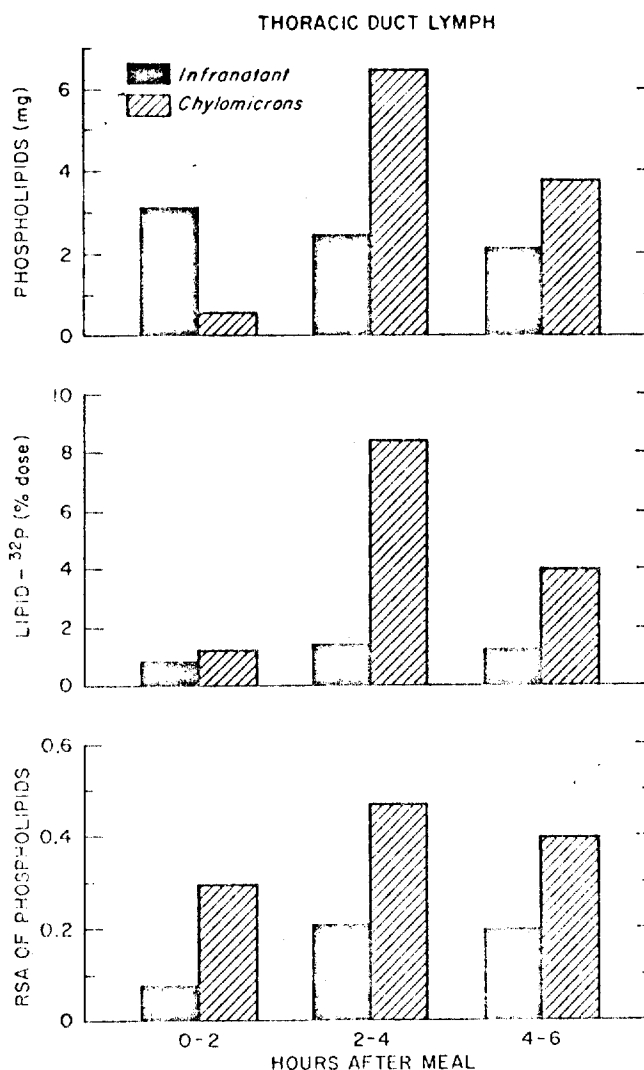


Fig. 1. Phospholipid and lipid- ^{32}P content of thoracic duct lymph of rats fed corn oil containing lecithin- ^{32}P . Rats 24, 25, and 26 were used in this study. The values given are the means of three experiments. RSA, relative specific activity.

RESULTS

Lecithin- ^{32}P —Incorporation of phospholipid of dietary origin into lymph chylomicrons was first studied in rats fed corn oil containing different amounts of lecithin labeled with ^{32}P (Table I). The total lipid content of the chyle collected for 6 hours was equal to 30 to 70% of that fed, whereas the lipid ^{32}P was 8 to 27%. The total phospholipid content of chyle, collected for 6 hours, ranged from 7 to 24 mg. Phospholipids constituted 5.9 to 11.8% of the lipid in chyle and 3.3 to 6.2% in chylomicrons. Increasing the lecithin content of the oil increased the amount of lipid recovered in chyle but had no effect on its phospholipid content. The relative specific activity of chylomicron phospholipids, however, increased directly with the lecithin content of the meal (Table II). The phospholipids in chylomicrons had a relative specific activity 2 to 3 times that in the infranatant fraction. When the lecithin content of the oil was between 6.5 and 11%, the relative specific activity of phospholipids in the chylomicrons averaged 0.37. These observations indicate that at least 37% of

the lipid phosphorus in chylomicrons can be derived from dietary lecithin if the lecithin content is high enough, at least 6.5%.

Distribution of phospholipid and lipid- ^{32}P between the chylomicron and infranatant fractions of lymph collected during three different intervals is shown in Fig. 1. The chylomicron fraction contained 0.5 mg of phospholipid in the first 2-hour collection, 6.5 mg in the second, and 3.7 mg in the third, whereas the infranatant fraction contained about the same amount, an average of 2.5 mg, in all collections. About 60% of the phospholipid collected during the 6-hour experiment was present in the chylomicron fraction and 40% in the infranatant fraction. The relative specific activity of phospholipids in the chylomicron fraction was more than twice that in the infranatant fraction in all collections. It should be noted that thoracic duct lymph samples, as collected in this study, included lymph not only from the intestines but also from other abdominal viscera and from the hindquarters of the animal. The chylomicron fraction presumably came only from intestines, whereas the infranatant fraction came from other tissues as well as intestines. The difference in relative specific activity between the two fractions may be due, at least in part, to dilution of labeled phospholipid from the intestines by unlabeled phospholipids from the other tissues.

1-Palmitoyl- ^3H , 2-linoleoyl- ^{14}C -lecithin- ^{32}P —The mode of incorporation of dietary phospholipids into lymph chylomicrons was studied in rats fed lecithin- ^{32}P containing palmitic acid- ^3H in position 1 and linoleic acid- ^{14}C in position 2 (Table III). The chylomicrons contained about 85% of the ^3H recovered in chyle. One-fifth of the ^3H in chylomicrons was present in lecithin and the rest was in neutral lipids. The ratio of ^3H : ^{32}P in chylomicron

lecithin was the same as that in the oil. This indicates that the dietary phospholipid incorporated into chylomicron lecithin retained intact its 1-acyl ester and glyceryl-phosphate bonds.

The chylomicrons also contained about 88% of the ^{14}C recovered in chyle (Table III). However, only 5% of the ^{14}C in chylomicrons was found in lecithin; the rest was in neutral lipids. The relative ratios of ^{14}C : ^{32}P and of ^{14}C : ^3H in chylomicron lecithin were 0.19 and 0.18, respectively; this suggests that one of the dietary phospholipid incorporated into chylomicron lecithin was absorbed as lecithin and four-fifths absorbed as neutral lipids. However, the very small amount of ^{14}C found in lecithin fraction, 5% of that in chylomicrons, could have been result of reacylation of lysolecithin with linoleic acid- ^{14}C . In that case, all of the phospholipid would have been absorbed as lysolecithin.

The relative composition of chylomicron lecithin collected during three different time intervals is shown in Table IV. The first 2-hour collection contained 6%, the second, 59%, and the third, 35% of the chylomicron lecithin collected during the experiment. The ratios of ^{32}P : P , ^3H : ^{32}P , ^{14}C : ^{32}P , and ^{14}C : ^3H in the lecithin were constant, an indication of uniform incorporation of dietary constituents throughout the experiment.

Lecithin Labeled with Choline- ^3H and ^{32}P (Diacylglycerolphosphoryl- ^{32}P -Choline- ^3H)—The fate of choline in lecithin was studied in rats fed corn oil containing the phospholipid labeled with choline-(methyl)- ^3H and ^{32}P (Table V). About 19% of the labeled choline fed was recovered in chyle lipids, and more than 80% of the latter was present in chylomicrons. The ratio of ^3H : ^{32}P in chylomicron lecithin was the same as that in the oil.

TABLE III

Recovery of ^3H and ^{14}C in lipids and ratios of ^3H : ^{32}P , ^{14}C : ^{32}P , and ^{14}C : ^3H in lecithin of thoracic duct lymph of rats tube-fed corn oil containing 1-palmitoyl-9, 10- ^3H , 2-linoleoyl-1- ^{14}C -lecithin- ^{32}P ^a

Rat No.	³ H recovered			¹⁴ C recovered			Relative ratio of ³ H: ³² P		Relative ratio of ¹⁴ C: ³² P		Relative ratio of ¹⁴ C: ³ H	
	Chyle total lipids	Chylomicron		Chyle total lipids	Chylomicron		Chyle total lipids	Chylomicron lecithin	Chyle total lipids	Chylomicron lecithin	Chyle total lipids	Chylomicron lecithin
		Total lipids	Lecithin		Total lipids	Lecithin						
	% dose			% dose			³ H: ³² P/ ³ H: ³² P in oil		¹⁴ C: ³² P/ ¹⁴ C: ³² P in oil		¹⁴ C: ³ H/ ¹⁴ C: ³ H in oil	
26	34.2	29.2	6.3	36.8	31.0	1.5	3.02	0.97	3.14	0.174	1.04	0.179
24	70.6	61.9	16.7	70.0	62.0	3.6	2.64	0.95	2.46	0.200	0.93	0.213
25	49.2	42.9	8.5	46.1	41.7	1.7	3.62	0.97	3.55	0.176	0.98	0.181

^a Lymph was collected for 6 hours.

TABLE IV

Constancy of ratios of ^{32}P : P , ^3H : ^{32}P , ^{14}C : ^{32}P , and ^{14}C : ^3H in lecithin of thoracic duct lymph of rats tube-fed corn oil containing 1-palmitoyl-9, 10- ^3H , 2-linoleoyl-1- ^{14}C -lecithin- ^{32}P ^a

Time	Chylomicron lecithin					
	Amount		Relative ratios			
	mg	% chylomicron lipid	^{32}P : P	^3H : ^{32}P	^{14}C : ^{32}P	^{14}C : ^3H
hrs			$(^{32}\text{P}:\text{P})/(^{32}\text{P}:\text{P})$ in oil	$(^3\text{H}:^{32}\text{P})/(^3\text{H}:^{32}\text{P})$ in oil	$(^{14}\text{C}:^{32}\text{P})/(^{14}\text{C}:^{32}\text{P})$ in oil	$(^{14}\text{C}:^3\text{H})/(^{14}\text{C}:^3\text{H})$ in oil
0-2	0.6 \pm 1.0	6.1 \pm 0.3	0.31 \pm 0.05	1.18 \pm 0.12	0.18 \pm 0.01	0.18 \pm 0.01
2-4	6.5 \pm 2.1	4.2 \pm 0.4	0.45 \pm 0.10	0.94 \pm 0.03	0.18 \pm 0.01	0.18 \pm 0.01
4-6	3.8 \pm 1.0	4.1 \pm 0.9	0.39 \pm 0.07	0.88 \pm 0.05	0.16 \pm 0.01	0.18 \pm 0.01

^a Rats 24, 25, and 26 were used in these studies. The values given are means \pm standard error. See Tables I, II, III, and Fig. 1 for other observations made in these animals.

TABLE V

Recovery of ^3H and relative ratio of ^3H : ^{32}P in lipids of thoracic duct lymph of rats tube-fed corn oil containing lecithin labeled with choline-(methyl)- ^3H and ^{32}P

Rat No.	^3H recovered			Relative ratio of ^3H : ^{32}P		
	Chyle total lipids	Infranatant total lipids	Chylomicron total lipids	Chyle total lipids	Chylomicron Total lipids	Lecithin
	% dose			^3H : ^{32}P / ^3H : ^{32}P in oil		
20	19.8	3.8	16.0	1.01	1.01	0.99
30	18.9	3.3	15.5	0.96	1.01	0.96
31	18.5	3.5	15.0	1.03	1.01	1.02

* Lecithin labeled with choline-(methyl)- ^3H and ^{32}P was isolated from soybean and diluted 1:5 with nonlabeled lecithin isolated from rat liver. Lymph was collected for 6 hours.

TABLE VI

Amount of ^{32}P recovered in lipid of thoracic duct lymph of rats tube-fed corn oil containing 1-acyl lysolecithin- ^{32}P

Lymph was collected for 6 hours.

Rat No.	Lysolecithin content of oil	Amount of oil fed	Chyle		Chylomicron		
			Total lipid	Lipid- ^{32}P	Total lipid	Lipid- ^{32}P	Lecithin- ^{32}P
	%	mg	mg	% dose	mg	% dose	
10	0.3	200	100	38	83	25	25
11	0.3	300	168	49	147	41	41
14	0.3	790	210	34	224	27	27
15	0.3	345	231	35	203	26	26

These findings indicate that the choline-phosphate bond remained intact in the dietary phospholipid which was incorporated into chylomicron lecithin.

Lysolecithin- ^{32}P . The findings presented above showed that most, if not all, of the chylomicron lecithin derived from dietary phospholipid was first hydrolyzed to 1-acyl lysolecithin and then reacylated. In order to determine whether dietary lysolecithin could be incorporated into chylomicron lecithin, rats were fed corn oil containing trace amounts of 1-acyl lysolecithin- ^{32}P (Table VI). The total lipid content of the chyle in these studies was equal to 30 to 68% of that of the oil fed. More than one-third of the lipid ^{32}P fed was recovered in chyle lipids. Incorporation of ^{32}P into chyle when lysolecithin- ^{32}P was fed was about twice that when trace amounts of lecithin- ^{32}P were fed (Table I, Rats 16 through 19). All of the ^{32}P in chylomicron lipid was found in lecithin; none was present as lysolecithin.

DISCUSSION

Earlier studies showed that phospholipids isolated from liver could be absorbed from the intestines and incorporated into lymph chylomicrons (1, 3). However, the phospholipids in the meal and in the chylomicrons were not identified. In the present experiments, purified lecithin, of either animal or plant origin, was fed and its incorporation into chylomicrons was followed by simultaneous measurement of two or more radioisotopes situated in different parts of the molecule. Conservation of the ratio of choline- ^3H : ^{32}P in the dietary phospholipid recovered in chylomicrons

makes it unlikely that the phospholipid was hydrolyzed to monoacyl phosphatidic acid during the process of being absorbed and incorporated into chylomicron lecithin. The finding that the ratio of palmitoyl- ^3H : ^{32}P in lecithin of lymph chylomicrons was the same as that in the lecithin fed could be interpreted as evidence that lecithin was absorbed intact. However, the marked fall in the ratios of linoleoyl- ^{14}C : ^{32}P and of linoleoyl- ^{14}C :palmitoyl- ^3H in lecithin recovered from chylomicrons of rats fed 1-palmitoyl- ^3H , 2-linoleoyl- ^{14}C -lecithin- ^{32}P indicates that some of the dietary lecithin was hydrolyzed to lysolecithin. The finding that the lecithin fraction contained 5% of the linoleic acid- ^{14}C in chylomicrons might suggest that only a small part of the lecithin was absorbed intact. On the other hand, the small amount of linoleic acid- ^{14}C in the lecithin fraction relative to that in the total lipid could be the result of reacylation of lysolecithin with linoleic acid. This possibility is supported by the studies of Whyte, Karmen, and Goodman (6) on the incorporation of free fatty acids into chylomicrons. They found that the lecithin fraction, also, contained 5% of the labeled linoleic acid recovered in chylomicrons when the labeled fatty acid was fed in a mixture of fatty acids (12% palmitic acid with 77% oleic and 10% linoleic, or with 12% oleic and 76% linoleic acids). The proportion of unsaturated to saturated fatty acids in this mixture was similar to that in corn oil. When labeled palmitic acid was fed as a free fatty acid, only 3% of the label recovered in chylomicrons was found in the lecithin fraction (6), whereas in the present experiments in which the label was fed as 1-palmitoyl- ^3H -lecithin, 25% was found in lecithin. This is additional evidence that the fed lecithin was hydrolyzed to 1-acyl lysolecithin before being incorporated into chylomicrons.

The anatomical site of hydrolysis of dietary lecithin was not determined. However, the findings that the intestines can hydrolyze lecithin to lysolecithin (7) and that fed lysolecithin is rapidly absorbed and incorporated into chylomicron lecithin (Table VI) suggest that hydrolysis occurs prior to absorption. Incubation studies have shown that slices of small intestines, of rat and hamster, also absorb and metabolize lysolecithin to lecithin (20). Rapid acylation of lysolecithin by small intestines has been demonstrated in rats injected intravenously with 1-palmitoyl- ^{14}C -lysolecithin (8).

The proportion of chylomicron lecithin which was derived from the test meal increased with the amount of lecithin fed. The maximum, however, was only 40%, even when the concentration of lecithin in the oil meal was 2 to 3 times that in the chylomicrons. This would mean, then, that at least 60% of the lecithin in chylomicrons came from endogenous sources. These sources could be lecithin in the bile (7, 21), lysolecithin and lecithin in the blood (8, 22), or lecithin synthesis *de novo* in the intestines (23). The relative importance of each has not been studied.

The results of the experiments presented above indicate that a major part of the lecithin molecule is conserved during the absorption of the phospholipid from intestines. Hydrolysis of lecithin to lysolecithin may be necessary for its transfer through the plasma membrane of the intestinal cell. This process is involved in the transfer of lecithin from blood to cells of other tissue (8). Conservation of the 1-acyl glycerylphosphoryl choline component of lecithin, of both endogenous and dietary origin, may be dependent on this ubiquitous cellular process.

Acknowledgments—The authors gratefully acknowledge the

excellent assistance of Miss Yedida Galanti and Mr. Gideon Hollander.

REFERENCES

1. ARTOM, C., AND SWANSON, M. A., *J. Biol. Chem.*, **175**, 871 (1948).
2. BLOOM, B., KIYASU, J. Y., REINHARDT, W. O., AND CHAIKOFF, I. L., *Amer. J. Physiol.*, **177**, 81 (1954).
3. BLOMSTRAND, R., *Acta Physiol. Scand.*, **34**, 147 (1955).
4. MARINETTI, G. V., ERBLAND, J., AND STOLTZ, E., *Biochim. Biophys. Acta*, **33**, 642 (1958).
5. MINARI, O., AND ZILVERSMIT, D. B., *J. Lipid Res.*, **4**, 424 (1963).
6. WHYTE, H. M., KARMEN, A., AND GOODMAN, D. S., *J. Lipid Res.*, **4**, 322 (1963).
7. BORGSTRÖM, B., *Acta Chem. Scand.*, **11**, 749 (1957).
8. STEIN, Y., AND STEIN, O., *Biochim. Biophys. Acta*, **116**, 95 (1966).
9. STEIN, Y., AND STEIN, O., *Biochim. Biophys. Acta*, **106**, 527 (1965).
10. LONG, C., AND PENNY, I. F., *Biochem. J.*, **65**, 382 (1957).
11. ROBERTSON, A. F., AND LANDS, W. E. M., *Biochemistry*, **1**, 804 (1962).
12. HÜTZL, J., CHATTERGEE, M., AND HERHAMMER, L., *Biochem. Z.*, **340**, 400 (1964).
13. FOLCH, J., LEES, M., AND SLOANE STANLEY, G. H., *J. Biol. Chem.*, **226**, 497 (1957).
14. NEWMAN, H. A. I., LIU, C. T., AND ZILVERSMIT, D. B., *J. Lipid Res.*, **2**, 403 (1961).
15. WAGNER, H., HERHAMMER, L., AND WOLFF, P., *Biochem. Z.*, **334**, 175 (1961).
16. BOLLMAN, J. L., CAIN, J. C., AND GRINDLAY, J. H., *J. Clin. Med.*, **33**, 1349 (1948).
17. STERN, I., AND SHAPIRO, B., *J. Clin. Path.*, **6**, 158 (1953).
18. BARTLETT, G. R., *J. Biol. Chem.*, **234**, 466 (1959).
19. OTT, D. G., RICHMOND, C. R., TRUJILLO, T. T., AND FORE, H., *Nucleonics*, **17**, 106 (1959).
20. NILSSON, A., AND BORGSTRÖM, B., *Biochim. Biophys. Acta*, **137**, 240 (1967).
21. PHILLIPS, G. B., *Biochim. Biophys. Acta*, **41**, 361 (1960).
22. NELSON, G. J., AND FREEMAN, N. K., *J. Biol. Chem.*, **235**, 1 (1960).
23. HÜBSCHER, G., SMITH, M. E., AND GURR, M. I., in R. M. DAWSON AND D. N. RHODES (Editors), *Metabolism and biological significance of lipids*, John Wiley and Sons, Inc., New York, 1964, p. 229.

Relationship between the Biliary Excretion of Bile Acids and the Excretion of Water, Lecithin, and Cholesterol in Man

T. SCHERSTÉN, S. NILSSON, E. CAHLIN, MARGARETA FILIPSON and GUNILLA BRODIN-PERSSON

Department of Surgery II, Sahlgrenska Sjukhuset, University of Göteborg, Göteborg, Sweden

Received: September 16, 1970

Abstract. The relationship between the biliary excretion of bile acids and the excretion of water, lecithin, and cholesterol was studied within a wide range of bile acid excretion rates in 5 patients operated on for nonobstructive gallstone disease. The bile acid excretion rate was varied by interruption of the enterohepatic circulation (EHC) and by duodenal refeeding of bile acids during interrupted EHC. A linear relationship between the bile acid excretion rate and the water excretion rate was found. In 2 of the patients the cholesterol excretion rate seemed to be independent of the bile acid excretion rate and in two of the remaining cases a linear relationship between

the excretion rates of these lipids was broken at bile acid excretion rates below 5 μ moles/min. The biliary excretion rate of lecithin was related to the bile acid excretion rate in a nonlinear way. This relationship suggests that the determining role of bile acids in the EHC for the biliary excretion of lecithin cannot be related primarily to the excretory mechanism in the hepatocytes. However, the findings are compatible with the hypothesis that the bile acids in the EHC affect primarily the synthesis of lecithin in the biliary excretion pool.

Key-words: Human bile, biliary excretion, bile flow, bile acids, bile lecithin, bile cholesterol.

The formation of bile is a complicated process and our knowledge of the mechanisms involved is still incomplete. However, during recent years considerable information has become available. It has been convincingly shown that bile production originates from the hepatocytic or canalicular excretion of organic anions, the subsequent osmotic diffusion of water and electrolytes and the hydraulic flow of water. The bile flow and the bile composition are then modified during the passage through the bile ductules and ducts.

The primary driving force for movement of water and other solutes into the canalicular lumen seems to be the hepatocytic excretion of bile acids [1-3].

The biliary excretion of lecithin, the dominant phospholipid in bile, and probably the excretion of cholesterol is dependent on the hepatocytic bile acid excretion [4-8]. However, the mechanism for this regulatory effect of bile acids is unclear. Swell *et al.* [6] have suggested that it is related to the formation of a specific macromolecular complex in the liver cell consisting of bile acids, cholesterol, lecithin and water and its subsequent excretion into the bile canaliculi. Some data in the literature contradict this hypothesis. Thus, it has been reported that the lipids in hepatic bile not necessarily appear in a micellar phase [9, 10]. Recently, we reported evidence that the bile acids in the enterohepatic circulation (EHC) exert a regulatory influence on the synthesis of lecithin in the biliary excretion pool in the liver [8]. However, the question of whether the bile acids in the EHC directly affect the synthesis rate of biliary lecithin or whether the synthesis rate is dependent primarily on the excretion rate of biliary lecithin could not be answered.

The purpose of the present work was to study the relationship between the biliary excretion of bile acids

and the excretion of water, lecithin and cholesterol further in order to obtain more insight into the regulatory effect of bile acids for the excretion of biliary lipids.

Subjects and Methods

Clinical Material. The study group comprised 5 patients, 1 woman aged 37, and 4 men, mean age 58.5. The patients were admitted to hospital for operation because of nonobstructive gallstone disease. They all had normal liver function according to the following definition: serum bilirubin, <1.0 mg per 100 ml; thymol turbidity, <0.10; alkaline phosphatase, <10 units (Buch); glutamic oxaloacetic transaminase, <30 units; and serum cholesterol, <300 mg per 100 ml. During the immediate preoperative period (1 month) none of them had taken any medicine known to affect the liver or had had acute cholecystitis or any other disease of known importance. The patients, who volunteered as subjects in the investigation, were all thoroughly informed about the details in the experiment and about the aim of the study.

Experimental. At the time of operation the common bile duct was cannulated as described previously [7].

The experiment was performed 7 days after the operation. Until this time the EHC was kept intact. This time for the experiment was chosen since the liver function is known to be restituted after the operation in about one week [11-13]. The patient was kept fasting during the 12 h period immediately before the study and while it was in progress. Early in the morning a duodenal catheter was inserted and the duodenal localization of the catheter tip was checked by X-ray investigation. At 8 a.m. the EHC was interrupted and then the hepatic bile was quantitatively

collected. Bile production was measured hourly and the bile composition was analysed. The bile acid excretion rate during the first hour was designated as the excretion rate during intact EHC. The patients, loss of water during the experiment was compensated for by infusion of Ringer's solution through the duodenal catheter. In some experiments (cases no. 1, 3 and 5) bile acids were refed at a rate of 16 μ moles/min. through the duodenal catheter in a concentration corresponding to the normal hepatic bile. Glycocholic acid (67%) and taurocholic acid (33%) (Sigma, grade II), the purity of which was analysed by thin-layer chromatography according to Gänshirt *et al.* [14], were given in Ringer's solution. The bile acid refeeding was started 5 hours after the interruption of the EHC and continued for 6 hours. The patients reported that they felt well and experienced no fatigue during the study.

Analytical. Bile acids were extracted, analysed and determined as described in detail in a previous paper [7].

Lipids were extracted from bile, separated and quantitated as described previously [15]. The phosphorus determination was performed following a procedure recently described by Svennerholm [16]. Cholesterol was measured by the method of Sperry and Webb [17].

Results

The relationship between the bile acid excretion rate and the excretion rate of water (bile flow), lecithin, and cholesterol can be seen in Figs. 1, 2 and 3.

In the two patients (cases nos. 3 and 5) illustrated in Fig. 1 bile acids were refed through the duodenal catheter 5 h after interruption of the EHC. This means that the excretion rate of bile acids was about 25 μ moles/min. at the start of the experiment and then successively decreased until a minimum value was reached after 4–5 h. Following duodenal refeeding of bile acids the biliary excretion rate of bile acids again increased.

A significant and linear relationship existed between the bile acid excretion rate and the rate of water excretion. A positive intercept on the ordinate can be seen when the regression lines are extrapolated to zero excretion rate of bile. The excretion rate of lecithin was also related to the bile acid excretion rate but this relationship was not linear. When these curves are extrapolated to zero bile acid excretion rate they intersect the ordinate at -0.72 and at $+0.59$, i.e. near *origo*.

In one of the patients (case no. 5) illustrated in Fig. 1 a significant and linear relationship existed between the rate of bile acid excretion and the excretion rate of cholesterol. In the other patient (case no. 3) no such relationship was seen.

In the patient (case no. 4) illustrated in Fig. 2 bile acids were not refed. Following interruption of the EHC the biliary excretion rate of bile acids decreased from about 25 μ moles/min. to a minimum level reached

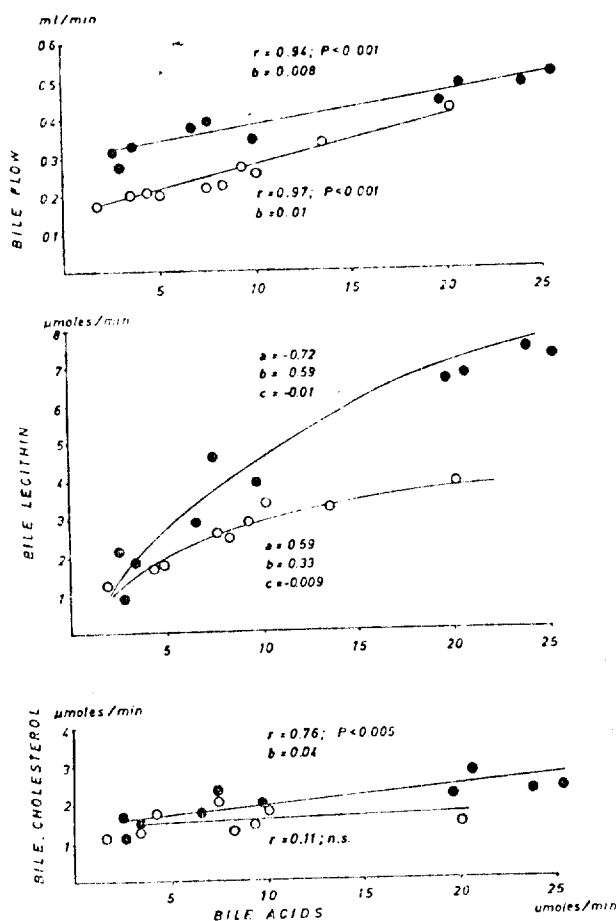


Fig. 1. The relationship between the biliary excretion rate of bile acids and the excretion rate of water (bile flow), lecithin and cholesterol in two patients (cases nos. 3 and 5). The bile acid excretion rate was varied by interruption of EHC and duodenal refeeding of bile acids during interrupted EHC. The relationships between the bile acid excretion rate and the excretion rates of water and cholesterol were calculated from the regression equation: $Y = k + b x$, where k represents the Y -intercept and b the regression coefficient. r = correlation coefficient. The relationship between bile acid excretion rate and the lecithin excretion rate was calculated from the equation $Y = a + b x + c x^2$. The coefficients a , b , c were calculated by the least square fit method.

after 5 hours whereupon the excretion rate was roughly constant throughout the remainder of the experiment.

A significant and linear relationship existed between the bile acid excretion rate and the excretion rate of water and cholesterol until the output of bile acids was below 5 μ moles/min. At this level the rate of excretion of water and cholesterol was independent of the excretion rate of bile acids. The same was found in the other patient (case no. 2) not refed with bile acids.

The biliary excretion rate of lecithin was related to the bile acid excretion rate in the same way as described

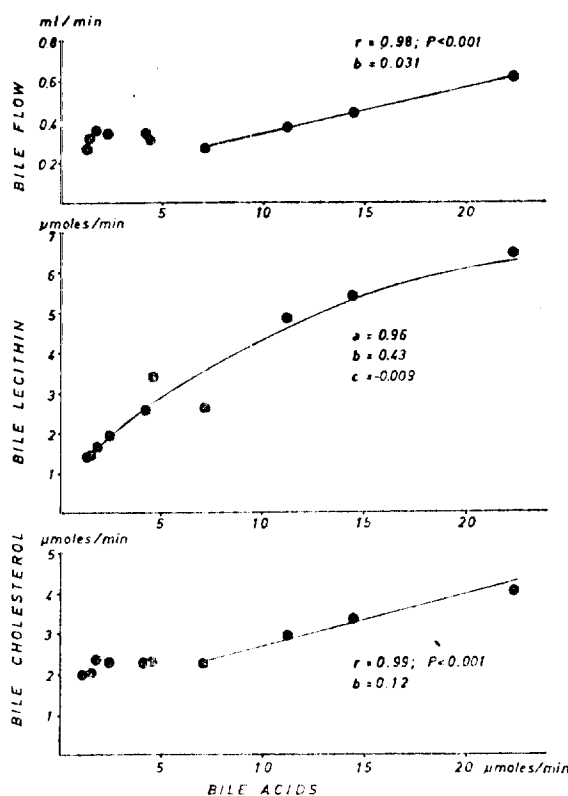


Fig. 2. The relationship between the biliary excretion rate of bile acids and the excretion rate of water (bile flow), lecithin and cholesterol in one patient (case no. 4). The bile acid excretion rate was varied by interruption of the EHC. The mathematical calculation were performed as indicated in Fig. 1

for the patients in Fig. 1. The Y-intercept (a) in this patient was 0.96.

In order to calculate the mean values of the bile-acid-dependent excretion rates of water, lecithin and cholesterol for the five patients all observations were plotted together (Fig. 3). At bile acid excretion rates below $5 \mu\text{moles/min}$, the water excretion rate and the cholesterol excretion rate were independent of the bile acid excretion rate in 2 out of the 5 patients. Because of that all values of water and cholesterol excretion rate at this low excretion rate of bile acids were excluded.

The mean value of the water excretion rate was $0.014 \text{ ml}/\mu\text{mole}$ bile acid. The mean excretion rate of bile acids during intact EHC was $15.1 \mu\text{mole/min}$, which means that the bile-acid-dependent excretion rate of water was 0.21 ml/min . The positive Y-intercept (k) corresponds to 0.20 ml/min .

The relationship between bile acid excretion rate and lecithin excretion rate was not linear. The positive Y-intercept (a) of the statistically calculated curve (see legend to Fig. 1) corresponds to $0.54 \mu\text{mole}$ lecithin/min.

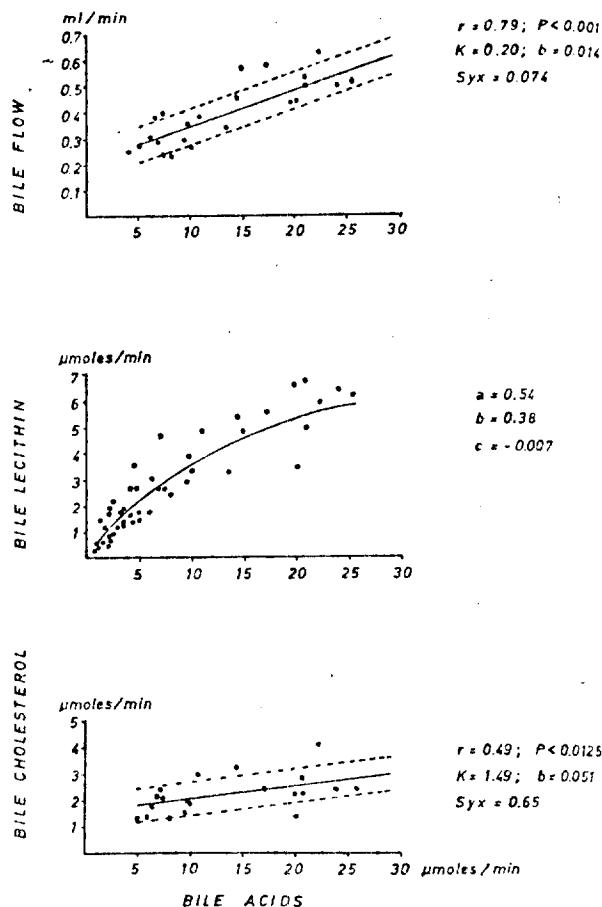


Fig. 3. The relationship between the biliary excretion rate of bile acids and the excretion rate of water (bile flow), lecithin and cholesterol in all five patients. Water and cholesterol excretion rates calculated only in relation to bile acid excretion rates higher than $5 \mu\text{moles/min}$. The mathematical calculations were performed as indicated in Fig. 1

The mean value of the bile-acid-dependent excretion rate of lecithin, obtained from the derivative of the curve, was $0.25 \mu\text{mole}/\mu\text{mole}$ bile acid.

The mean value of the cholesterol excretion rate was $0.051 \mu\text{mole}$ per μmole bile acid, which corresponds to $0.78 \mu\text{mole}$ cholesterol/min. during intact EHC. The positive Y-intercept (k) corresponds to $1.49 \mu\text{mole}$ cholesterol/min.

Discussion

In the present work the relationship between the biliary excretion of bile acids and the excretion of water, lecithin, and cholesterol in man was studied at different rates of bile acid excretion. The excretion rate of bile acids was varied by artificial interruption of the EHC and by duodenal refeeding of bile acids during interrupted EHC. This technique offers the possibility of studying these relations within a wide range of

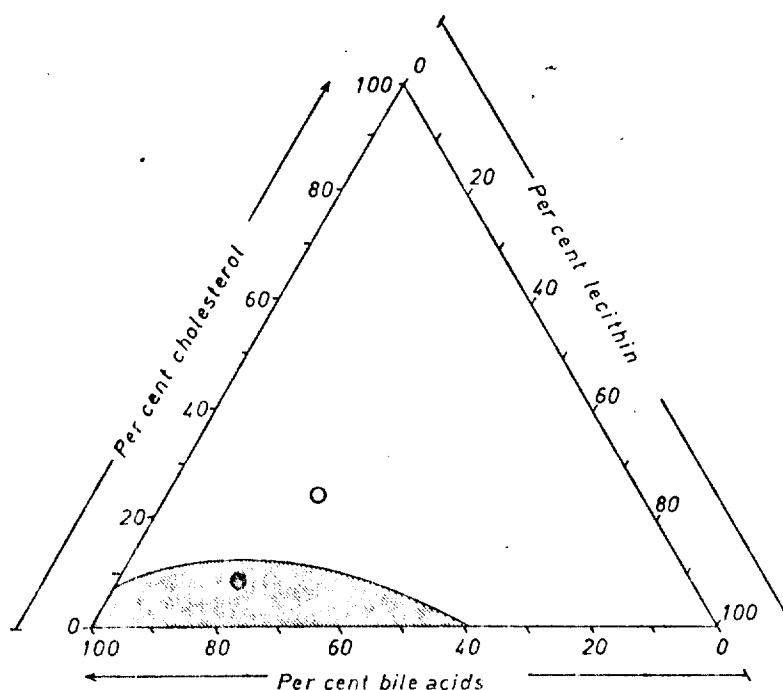


Fig. 4. The relative concentrations of bile lipids plotted in the triangular coordinates system according to Admirand and Small [19]. ● Bile acid excretion rates $> 15 \mu\text{moles/min.}$ (mean value of 9 observations); ○ Bile acid excretion rates $< 5 \mu\text{moles/min.}$ (mean value of 22 observations)

bile acid excretion rates. In a previous paper we showed that duodenal refeeding of bile acids during interrupted EHC had the same "normalizing" effect on the bile lipid composition as restitution of the enterohepatic circulation [7]. This finding indicates that the loss of water, electrolytes, lecithin, and cholesterol during a short-term interruption of the EHC does not affect the biliary excretory capacity of the liver. Hence, our experimental design seems to be adequate for this type of investigation.

The present study was performed on patients operated for nonobstructive gallstone disease. Recently, it has been suggested that the liver bile in patients with gallstone disease is abnormal with an increased concentration of cholesterol relative to the concentrations of bile acids and lecithin [18]. In our patients the lipid composition was normal as defined by Small and Rapo [18], i.e. the relative lipid composition of bile during intact EHC fell well inside the micellar zone when plotted according to Admirand and Small [19] (Fig. 4). In any case, it seems improbable that a changed lipid composition in gallstone patients as compared with normal patients should be dependent on a fundamentally different mechanism for the biliary excretion of lipids. Hence, the fact that our patients were operated on for gallstone disease probably does not influence the conclusions in the present work.

It has been shown in many species, including man, that the biliary excretion of water can be divided into two fractions, one dependent on the bile acid excretion rate, and one bile-acid-independent fraction [1-3]. In principle our findings tally well with these reports. In our material the two excretion fractions, calculated from the regression equation according to Preisig *et al.* [2], accounted for about the same amount of water excreted per unit of time. Preisig *et al.* [3] found the bile-acid-dependent fraction of water excretion to be $0.04 \text{ ml}/\mu\text{mole}$ bile acid and the bile-acid-independent fraction to be 0.15 ml/min. in 7 patients operated on for nonobstructive gallstone disease. Our corresponding figures were of the same order of magnitude.

In two of our patients (cases nos. 2 and 4) the linear relationship between the bile acid excretion rate and the water excretion rate was broken when the bile acid excretion was below $5 \mu\text{moles/min.}$, that is when the bile acid pool was almost depleted and only newly synthesized bile acids were excreted. At this low bile acid excretion level the water excretion rate was greater than the expected rate judged from the regression line (Fig. 2), suggesting that the bile-acid-independent water excretion rate was enhanced.

The bile-acid-independent water excretion has been ascribed to the ductular excretion of water [2, 21-24]. However, recent studies [20, 25, 26] have demonstrated that the bile-acid-independent water

excretion, besides this ductular excretion, might originate from the hepatocytes. With the methods used in the present work it is not possible to state whether the enhanced water excretion at low bile acid excretion rates in our cases was of ductular or hepatocytic origin. However, the increased water excretion was accompanied by an increased excretion rate of cholesterol in these two cases. A ductular excretion of cholesterol seems improbable considering the fact that secretin, which stimulated the ductular excretion [22], does not enhance the excretion rate of organic compounds [22, 27].

In the total material a significant correlation between bile acid excretion rates higher than 5 μ moles/min. and the cholesterol excretion rate was found (Fig. 3). However, in two of the patients (cases nos. 1 and 3) the cholesterol excretion rate seemed to be independent of the bile acid excretion rate. Although this independent relationship was not conclusive in one of these cases (case no. 3, Fig. 1), these results together with the fact that the cholesterol excretion rate was independent of the bile acid excretion rate when this rate was below 5 μ moles/min. suggest that the biliary excretion of cholesterol can occur independently of bile acids. These findings seem to argue against the theory that the biliary excretion of lipids is related to the formation in the liver cell of a specific macromolecular complex consisting of bile acids, cholesterol, lecithin and water [6]. A further argument against this hypothesis is that the relative composition of bile lipids in liver bile was outside the micellar zone (Fig. 4) when the bile acid excretion rates were below 5 μ moles/min, which means that the bile lipids can be excreted in a non-micellar form.

For the interpretation of the nonlinear relationship between the bile acid excretion rate and the lecithin excretion rate it is important to emphasize that our experiments were started at high rates of bile acid excretion, which then were decreased by interruption of the EHC and again increased by duodenal refeeding of bile acids. This means that the reason for the curve of lecithin excretion rate levelling off with the increasing rate of bile acid excretion can not be deficiency of lecithin in the biliary excretion pool in the liver. If the determining role of the bile acids in the EHC for the biliary excretion of lecithin was primarily related to the excretory mechanism this relationship thus should have been linear. Recently, we presented evidence that the synthesis of biliary lecithin is inhibited by interruption of the EHC and that duodenal refeeding of bile acids initiates a rapid increase in the synthesis rate [8]. This means that the bile acids in the EHC are a determining factor for the synthesis of biliary lecithin. The relationship between the bile acid excretion rate and the lecithin excretion rate found in the present study is in good agreement with that interpretation and it suggests also that the bile acids circulating in the EHC affect primarily the synthesis rate of biliary lecithin.

Acknowledgements. The investigation was aided by grants from the Swedish Medical Research Council and the Swedish Association against Cancer.

References

1. Sperber, I.: Secretion of organic anions in the formation of urine and bile. *Pharmacol. Rev.* 11, 109-134 (1959).
2. Preisig, R., Cooper, H., Wheeler, H.: The relationship between taurocholate secretion rate and bile production in the unanesthetized dog during cholinergic blockade and during secretin administration. *J. clin. Invest.* 41, 1152 (1962).
3. — Bucher, H., Stirnemann, H., Tauber, J.: Postoperative cholestasis following bile duct obstruction in man. *Rev. franç. Étud. clin. biol.* 14, 151-58 (1969).
4. Entenman, C., Holloway, R., Albright, M., Leong, G.: Bile acids and lipid metabolism. II. Essential role of bile acids in bile phospholipid excretion. *Arch. Biochem.* 130, 253-256 (1969).
5. Swell, L., Bell, C., Entenman, C.: Bile acids and lipid metabolism. III. Influence of bile acids on phospholipids in liver and bile of the isolated perfused dog liver. *Biochim. biophys. Acta (Amst.)* 164, 278 (1968).
6. — Entenman, C., Leong, G., Holloway, R.: Bile acids and lipid metabolism. IV. Influence of bile acids on biliary and liver organelle phospholipids and cholesterol. *Amer. J. Physiol.* 215, 1390 (1968).
7. Nilsson, S., Scherstén, T.: Importance of bile acids for phospholipid secretion into human hepatic bile. *Gastroenterology* 57, 525-532 (1969).
8. — — Influence of bile acids on the synthesis of biliary phospholipids in man. *Europ. J. clin. Invest.* 1, 109-111 (1970).
9. Verschuro, J.: Electro-chromograms of human bile. *Clin. chim. Acta* 1, 38-48 (1956).
10. Janiper, K., Jr.: Physicochemical characteristics of bile and their relation to gallstone formation. *Amer. J. Med.* 39, 98-107 (1965).
11. Rundle, S., Kass, M., Robson, B., Middleton, N.: Bile drainage after choledochostomy in man with some observations on biliary fistula. *Surgery* 37, 903-910 (1955).
12. Mollowitz, G.: Beobachtungen der Gallensekretion des Menschen. *Langenbecks Arch. klin. Chir.* 291 359-398 (1959).
13. Thureborn, E.: Human hepatic bile. Composition changes due to altered enterohepatic circulation. *Acta chir. scand. Suppl.* 303 (1962).
14. Gämshirt, H., Koss, S., Morianz, K.: Untersuchung zur quantitativen Auswertung der Dünnschichtchromatographie. *Arzneimittel-Forsch.* 11, 943-947 (1960).
15. Gottfries, A., Nilsson, S., Samuelsson, B., Scherstén, T.: Phospholipids in human hepatic bile, gallbladder bile, and plasma in cases with acute cholecystitis. *Scand. J. clin. Lab. Invest.* 21, 168-177 (1968).
16. Svennerholm, L.: Distribution and fatty acid composition of phosphoglycerides in normal human brain. *J. Lipid Res.* 9, 570-579 (1968).
17. Sperry, W., Webb, M.: A revision of the Schoenheimer-Sperry method for cholesterol determination. *J. biol. Chem.* 187, 97-106 (1950).
18. Small, B., Rapo, S.: Source of abnormal bile in patients with cholesterol gallstone. *New Engl. J. Med.* 283, 53-57 (1970).
19. Admirand, W., Small, D.: The physicochemical basis of cholesterol gallstone formation in man. *J. clin. Invest.* 47, 1043-1052 (1968).
20. Erdinger, S., Dhumeaux, D., Benhamou, J. P.: Effect on bile formation of inhibitors of sodium transport. *Nature (Lond.)* 223, 1276-1277 (1969).
21. Jorpes, J., Mutt, V., Jonson, G., Thulin, L., Sundman, L.: The effect of secretin on bile flow. *Gastroenterology* 45, 786-788 (1963).

22. Wheeler, H., Mancusi-Ungaro, P.: Role of bile ducts during secretin choleresis in dogs. *Amer. J. Physiol.* **210**, 1153 (1966).
23. Jones, R., Grossman, M.: The choleric response to feeding in dogs. *Proc. Soc. exp. Biol. (N.Y.)* **132**, 708-711 (1969).
24. Waitman, A., Dyck, W., Janowitz, H.: Effect of secretin and acetazolamide on the volume and electrolyte composition of hepatic bile in man. *Gastroenterology* **56**, 286-294 (1969).
25. Wheeler, H., Ross, E., Bradley, S.: Canalicular bile production in dogs. *Amer. J. Physiol.* **214**, 866-874 (1968).
26. Dhumeaux, D., Erlinger, S., Benhamou, J. P., Fauvert, R.: Effects of rose bengal on bile secretion in the rabbit: inhibition of a bile saltindependent fraction. *Gut* **11**, 134-140 (1970).
27. O'Maille, E., Richards, J., Short, A.: Factors determining the maximal rate of organic anion secretion by the liver and further evidence on the hepatic site of action of the hormone secretin. *J. Physiol. (Lond.)* **186**, 424-438 (1966).

T. Scherstén, M.D.
 Kirurgiska kliniken II
 Sahlgrenska Sjukhuset
 Göteborg, Sweden

Stecher, P.G. (Ed.) 1968
The Merck Index, An Encyclopedia of Chemicals and
Drugs, 8th Edition
Merck and Co., Rahway, New Jersey, p. 615

Effect of Feeding of "Soya Lecithin"* on Serum Cholesterol Level of Man.

ALFRED STEINER AND BEATRICE DOMANSKI. (Introduced by David Seegal.)

From the Research Service, First Division, Goldwater Memorial Hospital, Department of Hospitals, and College of Physicians and Surgeons, Columbia University, New York City.

In a previous report¹ it was postulated that the hypercholesterolemia resulting from the feeding of egg yolk powder, which is rich in cholesterol and lecithin, was due partially to its lecithin content. It was therefore considered of interest to determine the effect of feeding "soya lecithin" on the serum cholesterol level of 8 human subjects.

Method. Twenty-five grams of "soya lecithin"* were fed daily to 7 patients for 6-week periods and to one patient (No. 5) for a 10-week period. The "soya lecithin" was fed in 5 g portions spread between two crackers. The diagnosis in 6 of the 8 patients was coronary arteriosclerosis, made on the basis of clinical symptoms, signs and electrocardiographic changes. Five of the 6 individuals had previously experienced a myocardial infarction. The remaining 2 patients had classical rheumatoid arthritis, the diagnosis of which was confirmed by roentgenographic change. No untoward symptoms or signs occurred during the "soya lecithin" feeding periods. Serum cholesterol determinations by the method of Bloor, Pelkan, and Allen² were made twice weekly during an initial 4-week control period, the 6-week "soya lecithin" feeding period and a subsequent 4-week control period. Throughout these observations, the patients were fed a standard hospital diet. This was not rigidly controlled. It contained approximately 90 g of protein, 100 g of fat, and 340 g of carbohydrate, with a caloric equivalent of 2600 calories. Four of the patients were fed "soya lecithin" for a

second test period of 6 weeks. Basal metabolic rates were determined during the control period, and in the 4th and 5th week of the "soya lecithin" feeding period.

Results. The results of feeding "soya lecithin" on the serum cholesterol level are contained in Table I. There were 12 feeding periods in 8 patients. It can be seen that the serum cholesterol level declined during each period of "soya lecithin" feeding. The extent of the fall from the average of control period determinations varied from 44 to 144 mg/100 cc. Fig. 1 demonstrates, in one instance, the lowering effect of "soya lecithin" on the serum cholesterol level. The serum cholesterol, however, returned to the control levels, in each instance, after 4 to 5 weeks, even though the "soya lecithin" feeding was continued. After further control observations, 4 of the patients were fed "soya lecithin" during a second period. The results obtained were similar to those in the initial experiment. Basal metabolic rates remained approximately the same during the control period and the period of maximum depression of the serum cholesterol level.

Discussion. From the above it is evident that the feeding of "soya lecithin" to human subjects results in a lowering of the serum cholesterol level. Corwin³ has reported that a hypercholesterolemia was produced in dogs by the feeding of lecithin derived from the adrenal gland. However, Kesten and Silbowitz⁴ have stated that the feeding of "soya lecithin" inhibits the hypercholesterolemia of rabbits fed a diet rich in cholesterol. The effect of "soya lecithin" and that of lecithin obtained from the adrenal gland on serum cholesterol level are apparently different. It should be emphasized that "soya lecithin" is

¹ Steiner, A., and Domanski, B. N., *Am. J. Med. Sc.*, 1941, **201**, 820.

* American Lecithin Co., Elmhurst, L.I. Approximate composition, lecithin 20%, cephalin 20%, oil 30%, phytosterols 2%, inositol and allied compounds 15%, carbohydrates 10%.

² Bloor, W. R., Pelkan, K. F., and Allen, D. M., *J. Biol. Chem.*, 1922, **52**, 191.

³ Corwin, W. C., *Arch. Path.*, 1938, **26**, 426.

⁴ Kesten, H. D., and Silbowitz, R., *Proc. Soc. Exp. Biol. and Med.*, 1942, **49**, 71.

"SOYA LECITHIN" ON SERUM CHOLESTEROL LEVEL

237

TABLE I.
Effect of Soya Lecithin Feeding on Serum Cholesterol Level of 8 Patients.
Serum cholesterol expressed as mg/100 cc.

Patient No.	Initial control period (4 wks)		Soya lecithin period (6 wks)		2nd control period (4 wks)	
	Range	Avg	Lowest value	Ext. of decline	Range	Avg
1	329-378	349	290	59	309-357	340
2	537-561	546	402	144	458-536	496
3	458-505	474	388	86	442-462	456
4a	320-386	348	275	73	315-345	328
b	345-380	365	302	63	371-400	384
†5a	242-252	245	192	53	238-258	245
b	239-256	245	195	50	236-256	242
*6a	226-240	235	190	45	215-245	235
b	240-252	245	201	44	253-275	262
*7	232-238	235	180	45	220-238	232
8a	416-424	420	344	84	395-426	410
b	390-420	410	335	75	416-449	430
Avg 68						

* Patients with rheumatoid arthritis.

a = First experimental period.

b = Second " " "

† Lecithin feeding period—10 weeks.

EFFECT OF FEEDING 25 GMS. OF SOYA LECITHIN ON THE
SERUM CHOLESTEROL LEVEL - PATIENT #5

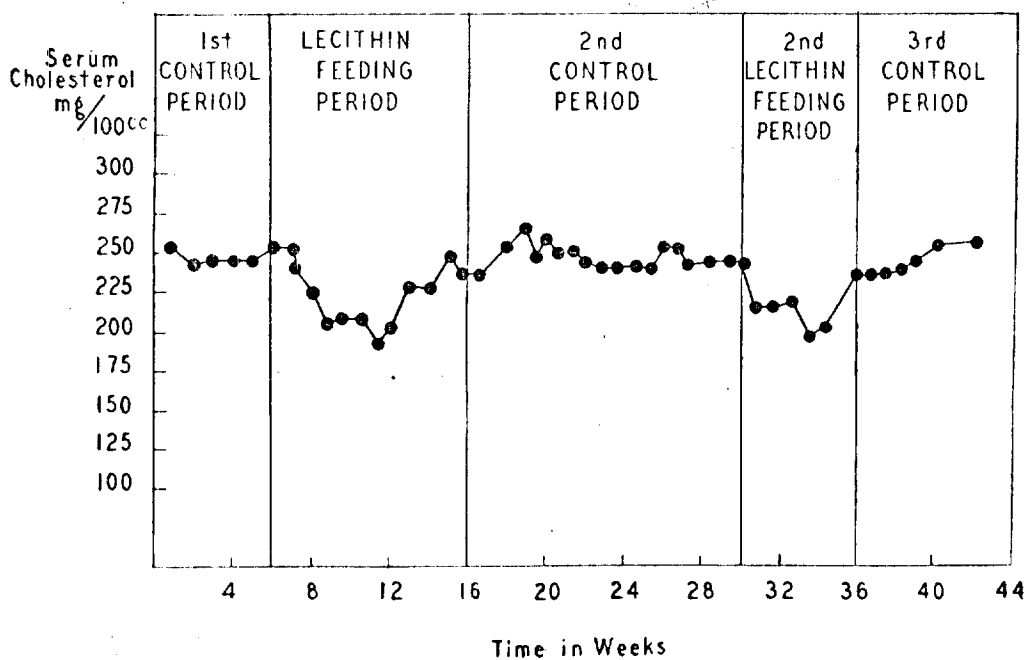


FIG. 1.

composed not only of lecithin, but also of cephalin, oil, phytosterol, inositol, and carbohydrates as well.

The inference that the hypercholesterolemia resulting from the feeding of egg yolk powder was partially due to its lecithin content¹ is not supported by these experiments. On the contrary, "soya lecithin" has been found to lower the serum cholesterol level not only in people on a normal diet but also in rabbits on a high cholesterol regime.⁴

Since this article was prepared Gross and Kesten⁵ have reported that the feeding of "soya lecithin" was effective in reducing the hypercholesterolemia in all of 3 patients. The diagnosis in 2 of these individuals was xantho-

matosis. *Necrobiosis lipoidica diabetorum* was the stated diagnosis in the third patient. Adlersberg and Sobotka⁶ have also shown that the elevated serum cholesterol of 5 patients with xanthomatosis was lowered by "soya lecithin" feeding.

Summary. (1) Twenty-five grams of "soya lecithin" were fed to 8 patients for 6-week periods. (2) The serum cholesterol level in each instance was lowered significantly. The decline was maintained for only 5 weeks despite the maintenance of the "soya lecithin" regime. (3) The induced hypocholesterolemia was not associated with an increase in the basal metabolism.

¹ 5 Gross, P., and Kesten, B., *Arch. Derm. and Syph.*, 1943, **47**, 159.

⁶ Adlersberg, D., and Sobotka, H., *J. Mt. Sinai Hosp.*, 1943, **9**, 955.

Exogenous Emulsifiers and Fat Absorption.* (19764)

HERBERT C. TIDWELL AND MARY E. NAGLER.

From the Department of Biochemistry, Southwestern Medical School of The University of Texas, Dallas.

The rather general acceptance in the recent past of Frazer's(1) partition hypothesis seems to have strengthened the idea that emulsifying agents should hasten the rate of the absorption of fat from the intestine. Such an effect might be the result of a greater emulsification which may either promote the speed of absorption of the finely divided particulate fat or permit a more rapid action of the lipolytic enzymes.

The findings of several investigators could be interpreted as evidence supporting this effect of emulsifiers. An increased rate of fat absorption has been obtained when the ingested fat is supplemented with lecithin(2-4), which has an emulsifying action. Becker *et al.*(5) reported Tween 80 to affect fat absorption differently in young and aged people, but their results seemed to indicate a faster rate of fat absorption in both groups. Tween 80 has been found by Jones *et al.*(6) to promote the absorption of fat and fat soluble substances by patients with steatorrhea. However, they stated that in the normal subject "the absorption of dietary fat is so nearly complete that the addition of such an agent to the diet would seem to be of negligible value." An increased rate of fat absorption has also been reported to occur when an emulsified fat is instilled in the ligated rat intestine.

Earlier studies of Holt *et al.*(8) gave no evidence that the normal infant found any difficulty in intestinal emulsification of dietary fat. The results of Jones *et al.* were not confirmed by Badenoch, Johnson and their collaborators(9) and the latter group reported Tween 20 to be ineffective in reducing the fecal fat of prematures and of infants with steatorrhea. Recently, Shoshkes *et al.*(10) found absorption of fat to be unaffected by the presence of 20% lecithin or Tween 80, amounts greatly in excess of that required to

make excellent emulsions by mechanical means.

With this somewhat conflicting evidence, it seemed wise to include some of the synthetic emulsifiers in our studies of the various factors influencing the rate of fat absorption. The effect of a monoglyceride† and of a number of the fatty acid esters of some sorbitol or alkylene oxide derivatives‡ on this rate has been determined at different levels of fat intake and emulsifying supplement. Several of the latter emulsifying agents were included in these studies because they have slightly different physical properties. Also an attempt has been made to learn whether these substances influenced either gastrointestinal motility or fat splitting, since such actions might affect the rate of fat absorption.

Experimental. The groups of animals used were all male or female albino rats with weights ranging from 150 to 500 g. As checks upon each other, two methods, the chylomicrograph and a modified Cori technic, were used to determine the rate of fat absorption.

A number of investigators(4,5,11,12) have shown the former method can be used to determine the relative amount of fat in the blood and that the area under the curve obtained gives a similar measure of the rate of the various processes involved. After a 24-hour fast, the rats were given 0.05 ml of olive oil per square decimeter of body surface with or without the supplement of 6 or 20% of the emulsifier. The small amount of fat permit-

† Generously supplied by Distillation Products, Inc., Rochester, N. Y.

‡ Atlas Powder Company trade marks and chemical descriptions:

Tween 80—polyoxyethylene (20) sorbitan mono-oleate

Myrj 45—polyoxyethylene (8) stearate

Span 60—sorbitan monostearate

Tween 60—polyoxyethylene (20) sorbitan monostearate

* The funds for this study were provided in part through the courtesy of The Atlas Powder Co., Wilmington, Del.

EXOGENOUS EMULSIFIERS AND FAT ABSORPTION

13

TABLE I. Effect of Supplements of Various Emulsifiers on Blood Fat Levels after Fat Ingestion (Chylomicrographs).

No. rats	Fat supplement	Chylomicron count				Area under curve	"t" value
		Fast-ing	1	1½	2		
8	Saline	13	36	41	42	44	83 ± 6
10	Tween 80 6%	15	41	47	46	45	91 ± 3
8	" 20%	8	47	44	51	53	98 ± 6
10	Saline	6	69	43	44	40	92 ± 5
	Myrj 45 6%	4	58	51	49	36	95 ± 5
	Span 60 6%	4	63	48	51	49	102 ± 8
	Tween 60 6%	5	64	46	45	55	100 ± 5
	Monooleate 6%	4	66	55	41	44	97 ± 8
35	Saline	8	48	52	52	44	99 ± 4

Including the stand. error of the mean and Fisher's "t" value for areas under the curve.

ted a more accurate counting of the chylomicrons and the use of a more physiological intake of fat. The supplements given approximated the levels used by Jones, Becker, and Stare in their studies. The method followed was that previously used in this laboratory(4). An eyepiece micrometer disc (5 mm square divided into 100 squares) replaced the Whipple disc, and from the 4 or more fields of 100 squares counted, the average number of chylomicrons in 10 squares were the counts recorded.

The second method of this study made use of the Cori technic as modified by Deuel *et al.*(13). The determination of the rate of fat absorption by a recovery of unabsorbed fat after an interval for absorption has been found satisfactory by a number of investigators(7,14). The ages or sex of the animals did not influence this absorption rate. The rats were fasted 48 hours and given by tube 0.15 or 0.30 ml of Sudan IV stained olive oil per square decimeter of body surface. After a 2- or 3-hour period for absorption the rats were sacrificed and the distance the stained fat had passed along the intestine as well as the total intestinal length measured. Finally, the fat was recovered, weighed and the free fatty acids titrated. This procedure permitted not only an estimation of the per cent of fat absorbed and the rate of this process, but also a relative measure of gastrointestinal motility and fat splitting as affected by the various supplements given.

Results and discussion. The chylomicrographs, obtained in 2 experiments when the fat was given alone or with either 6 or 20%

of the exogenous emulsifiers, are shown in Table I. Although the area under the curve for the first group receiving only the fat appears to be much smaller than that of those receiving the 20 or 6% of Tween 80, the differences are not statistically significant. Wide individual differences in this particular control group (fat alone), and a mean control value of 99 on 35 rats involved in all studies about this time, would indicate that the differences should not be significant. A later experiment confirmed this conclusion. That similar results were obtained when supplements of 6% Myrj 45, Span 60, Tween 60 and glyceryl monooleate were given with the fat may be seen in Table I. The similar peaks and areas under the curves indicated comparable fat absorption in all groups. Hence, the use of this method gave no evidence of any change in the rate of the absorption of the fat when it was supplemented with any of the emulsifiers.

The results obtained by the modified Cori technic are included in Table II. In the first experiment, there was no significant change in the per cent of fat absorbed or in the rate of its absorption over a 2-hour period when 0.15 ml of fat per square decimeter of body surface was supplemented with 20% Tween 80 or glyceryl monooleate. Similar results were obtained when twice as much fat was supplemented with 6% of the previously mentioned exogenous emulsifiers and the absorptive period extended to 3 hours. The latter changes permitted a comparison of our values with those of Deuel and co-workers when they fed cottonseed oil. Good agreement was ob-

TABLE II. Absorption and Splitting of Fats, and Intestinal Motility as Affected by Various Supplements of Emulsifiers (by Recovery).

No. rats	Fat supplement	Fat absorbed			% of free fatty acids recovered	% of length of intestine traversed
		%	mg/dm ² /hr	"t" value		
16*	Saline	33.9	21.6 ± 1.7	~	17.5	80
8*	Tween 80 20%	32	22.9 ± 1.6	1.067	—	90
8*	Monoolcate 20%	36.8	25.6 ± 2.5	2.068	16.5	81
8	Saline	44.3	40.3 ± 2.9		17	85
8	Myrj 45	47.8	43.9 ± 3	.860	15.9	94
9	Span 60	48.8	44.6 ± 1	1.263	12	85
7	Tween 60	42.3	39 ± 3.6	.284	12.5	92

* 2 hr absorptive period after ingestion of .15 ml of fat per dm² of body surface; all others, 3 hr and .30 ml of fat.

served. Again, there was no evidence of an effect upon the rate of fat absorption when it was determined by the recovery method instead of from changes in the blood fat levels.

The amounts of free fatty acids in the recovered fat and the relative length of the intestine traversed appear to be unaffected by the presence of these amounts of the emulsifiers. These results are in agreement with previous, and as yet unpublished, data from similar tests. Thus, the evidence suggests no change in the fat splitting or intestinal motility in the animals given the fat and supplement, as compared with a control group receiving the fat alone.

It appears important that it be established whether the physical effect of an increased emulsification of the fat in the intestine of the normal animal plays a role in promoting fat absorption. Our results from the use of 2 methods are in complete agreement with those from Stare's laboratory—that the absorption of orally fed fat is unaffected by the presence of emulsifiers, even when greatly in excess over that needed to make excellent emulsions by mechanical means. Factors, such as splitting and fat passage along the intestine, that might affect such absorption also appear unchanged. It seems logical to suppose that under normal conditions, a sufficient emulsification of the fat would naturally occur in the normal intestine to permit the maximum absorption of the fat. If this be true, then use of exogenous emulsifiers as fat supplements should not affect the rate of fat absorption in the normal subject. Such reasoning is in accord with our findings.

If it be established that emulsifier supplements do not promote fat absorption, then the

effect of lecithin must be otherwise explained. The fact that both lecithin and choline(4) appear to increase the rate of fat absorption would suggest that one or both are involved in some chemical mechanism required for fat absorption. Thus it appears that the presence of substances that may promote changes in the physical state of the fat do not increase the rate of the absorptive process in the normal animal, while those that may enter into the chemical actions involved do promote fat absorption.

Summary. As determined by changes in the blood fat levels and by recovery of fat from the intestine after an absorptive period, the rate of fat absorption in normal, orally fed rats appears to be unaffected by 6 or 20% supplements of a number of exogenous emulsifiers. The lack of changes in the degree of fat splitting and intestinal motility which could affect fat absorption are in complete agreement with the above finding. The action of lecithin in promoting fat absorption seems best explained by other than its emulsifying action.

The technical assistance of Mrs. Anne M. Miller is acknowledged.

1. Frazer, A. C., *Physiol. Rev.*, 1946, v26, 103.
2. Augur, V., Rollman, H. S., and Deuel, H. J., Jr., *J. Nutrition*, 1947, v33, 177.
3. Adlersberg, D., and Sobotka, H., *J. Nutrition*, 1943, v25, 255.
4. Tidwell, H. C., *J. Biol. Chem.*, 1949, v182, 405.
5. Becker, G. H., Meyer, J., and Necheles, H., *Gastroenterology*, 1950, v14, 80.
6. Jones, C. M., Culver, P. J., Drummey, G. D., and Ryan, A. E., *Ann. Int. Med.*, 1948, v29, 1.
7. Shoshkes, M., Geyer, R. P., and Stare, F. J., *J. Lab. and Clin. Med.*, 1950, v35, 968.

GROWTH HORMONE IN NORMAL MEN

8. Holt, L. E., Jr., Tidwell, H. C., Kirk, C. M., Cross, D. M., and Neale, S., *J. Pediatrics*, 1935, v6, 427.
9. Badenoch, J., Truelove, S. C., and Ward-McQuaid, J. N., *The Lancet*, 1951, v260, 508; Johnson, A. L., Scott, R. B., and Newman, L. H., *Am. J. Dis. Child.*, 1950, v80, 545.
10. Shoshkes, M., Geyer, R. P., and Stare, F. J., *Proc. Soc. Exp. Biol. and Med.*, 1950, v75, 680.
11. Elkes, J. J., Frazer, A. C., and Stewart, H. C., *J. Physiol.*, 1939, v95, 68.
12. Cooper, J. J., and Lusk, H., *Am. J. Digest. Dis.*, 1942, v9, 395.
13. Deuel, H. J., Jr., Hallman, L., and Leonard, A., *J. Nutrition*, 1940, v20, 215.
14. Irwin, M. H., Steenbock, H., and Templin, V. M., *J. Nutrition*, 1936, v12, 85.

Received July 29, 1952. P.S.E.B.M., 1952, v81.

JULY 1971

The American Journal of Surgery

VOLUME 122

NUMBER 1

EDITORIAL**Current Status of Investigations into the Etiology of Gallstones**

RONALD K. TOMPKINS, MD, Los Angeles, California

Remarkable advances in biochemical discoveries and technics over the last decade make it rational to predict that the solution of the problem of gallstones in man, literally as well as figuratively, can be accomplished in the next few years. Surgeons have both a unique scientific opportunity and a moral obligation to work toward the realization of this goal. As an example, Maki has recently advanced a biochemical explanation of the formation of calcium bilirubinate stones, the most common type found in the rural Japanese population. He has shown that B-glucuronidase from bacteria frees bilirubin from its glucuronide and allows it to combine with calcium to form a precipitate in bile. Clinically, he has observed that virtually all patients with this type of gallstone have bile infected with the B-glucuronidase producing bacteria.

There is now no doubt that the events which lead to formation of biliary tract stones are biochemical alterations in bile which lead to the insolubility of one or more of the normal biliary constituents such as cholesterol, calcium, or bile pigments. In this country and others of western culture, over 90 per cent of all gallstones are composed of cholesterol, either "pure" or combined with bile pigments. The most fruitful studies of cholesterol stone formation have been those directed toward discovering the factors which normally maintain this lipid in an aqueous bile solution. Isaksson, in 1954, demonstrated that bile

salts alone could not solubilize all the cholesterol in a given bile sample. He concluded that biliary phospholipids (chiefly phosphatidyl choline or "lecithin") were necessary to aid the bile salts in cholesterol solubility. Subsequent studies of model bile solutions have shown that the solubility of cholesterol is approximately eight times greater, mole per mole, in lecithin solutions than in solutions of bile salts.

The clinical importance of high biliary lecithin concentrations has been emphasized by our finding that patients with cholelithiasis have only one third the level of gallbladder bile lecithin that is found in human subjects with normal biliary systems. Since the cholesterol concentrations in these two groups are similar, normal human subjects have a biliary phospholipid:cholesterol (P/C) ratio which is three times greater than that found in patients with cholesterol gallstones. The reason for this discrepancy is not yet known. Further *in vivo* evidence of lecithin's role in solubilizing cholesterol is the observation that cholesterol gallstones in man dissolve at an average rate of 17 mg per day in the dog's gallbladder, where the bile has a P/C ratio twelve times greater than that in the normal human subject.

Theoretically, it may seem an oversimplification to attempt to explain the formation of gallstones on changes in only one of the many biliary constituents. However, this hypothesis has led to practically productive research. Lecithin addition to diets low in fat and cholesterol has resulted in the elevation of P/C ratio of hepatic bile obtained from our patients with T tubes in place. This phospholipid-rich bile has shown the ability to hold more cholesterol in solution than bile from

From the Department of Surgery, University of California School of Medicine, Los Angeles, California. This work was supported by a grant from the John H. Hartford Foundation, Inc, New York, New York.

6 Editorial

the same patient before lecithin feeding. Feeding studies in animals, using labeled lecithin, have demonstrated that a substantial portion of the phospholipid is transported intact via thoracic duct lymph chylomicra to the systemic circulation. Other laboratory studies indicate that labeled lecithin appears a few hours later in the common duct bile. The oral administration of lecithin to human subjects has been without complications and generally well tolerated. Future efforts should be directed to making the diet as rich in lecithin as possible while not palling to the appetite. Further metabolic studies may show that feeding precursors of biliary lecithin synthesis, rather than crude lecithin, will be more effective in elevating the P/C ratio.

The importance of discovering a nontoxic, easily tolerated method of increasing the cholesterol-dissolving capacity of human bile, such as that of feeding lecithin, is evident. It may be some time before elective surgical removal of gallstones, with its low morbidity and mortality, is replaced by chemical or dietary therapy. However, there is an immediate need for a meaningful alternative to surgical treatment for a large number of patients with gallstones. These are the unfortunate per-

sons who are considered not to be suitable candidates for operation because of coexistent heart disease, obesity, personal preference, or other medical reasons. Although it is difficult to estimate the number of persons in this group, it must be sizable.

It is also possible that such dietary modifications would have preventive value in those persons in whom the development of cholelithiasis is more likely. Patients undergoing vagotomy and gastric drainage procedures, diabetic males, and women with multiple pregnancies as well as those taking oral contraceptives have been indicated to be at risk in this regard.

Nobel-laureate Dr Frances Peyton Rous once characterized gallstone research as "top-heavy with hypotheses and dismal with uncorrelated observations." Surgeons trained in the basic sciences of physiology and physiologic chemistry, by virtue of their direct access to the human biliary tract and its secretions, are now in a unique position to correlate these observations with well planned and executed clinical experiments. To these investigators, the challenge of reducing the necessity for surgical removal of gallstones should be irresistible.

Clin. Res. 21:276, 1973

INDUCED ALTERATIONS IN HUMAN SERUM LIPIDS BY
PROLONGED PHOSPHOLIPID INGESTION. Ronald K.
Tompkins, Richard F. Corlin*, Lillie Grossman
Parkin*, and William King, III*. UCLA Schools of
Medicine and Public Health, Los Angeles, Calif.

The effects of chronic administration of phospholipids to humans with gallstone disease have been the subject of study in this laboratory for over one year. In addition to monitoring hepatic, renal, cardiovascular function tests and biliary chemistries, serial determinations of fasting serum cholesterol (C), phospholipids (PL), triglycerides (TG) and lipoprotein electrophoreses were carried out. Following a control period of two months on a basic low fat diet, 48 grams a day of commercial soybean lecithin was added to this diet in six patients.

Serum C decreased from a control mean value of $240.3 \text{ mg\%} \pm 49.9 \text{ (S.D.)}$ to $189.8 \pm 20.8 \text{ mg\%}$ after 8 mos. on lecithin. Serum TG decreased from $148.8 \pm 39.4 \text{ mg\%}$ to $114.0 \pm 38.7 \text{ mg\%}$ and serum PL increased from $210.3 \text{ mg\%} \pm 48.8$ to $227.3 \pm 43.7 \text{ mg\%}$ during the same period. Three male patients began the study with abnormal lipoprotein electrophoreses (2 Type IV and one Type II) and these patterns were unaffected by the low fat diet period. 4 mos. after lecithin was begun, however, all lipoprotein electrophoreses became normal and remained so for the duration of the study period. No hepatic, renal or cardiovascular changes occurred during the study.

The data demonstrate that long-term ingestion of large amounts of soybean lecithin is well-tolerated. This diet has been associated with lowering of serum C and TG levels and a return of lipoprotein electrophoreses to normal in the patients studied.

J. Clin. Invest. 51(6) 1337-1350

Biliary Excretion of Lecithin and Cholesterol in the Dog

HENRY O. WHEELER and KATHERINE K. KING

*From the Department of Medicine, University of California, San Diego,
School of Medicine, La Jolla, California 92037*

ABSTRACT The biliary excretion rates of bile acid, lecithin, and cholesterol were measured in unanesthetized dogs after interruption of enterohepatic circulation and during infusions of sodium taurocholate, sodium glycocholate, sodium dehydrocholate, SC2644 (a bicyclic organic acid with high choleretic potency), and secretin. Both lecithin output and cholesterol output were directly related to bile acid excretion rate. The curves describing these relationships were concave downward. Molar concentration ratios of lecithin-to-bile acid declined gradually from approximately 0.4 to 0.2 as bile acid output increased from approximately 1 to 70 μ moles/min. Cholesterol-to-lecithin molar ratios were highest (0.05-0.15) at very low rates of bile acid excretion, but descended rapidly to a plateau (0.03-0.04) which was constant over the entire range of bile acid excretion rates from 10 to 70 μ moles/min.

Similar lipid excretion patterns were observed during glycocholate infusion, but secretin-induced choleresis and dehydrocholate-induced choleresis were unaccompanied by any increments in lecithin or cholesterol excretion and SC2644 (which caused a marked increase in canalicular bile production as measured by erythritol clearance) caused a depression of lipid excretion.

The data are consistent with the view that lecithin moves passively from cell membranes to intracanalicular micelles, that transport of cholesterol is coupled to lecithin transport, and that there is also a small amount of independent passive transport of cholesterol from membranes to micelles. A model developed on these assumptions has been shown to behave in a fashion consistent with the entire range of these observations.

INTRODUCTION

Two highly insoluble lipids, cholesterol and lecithin, are present in bile in appreciable concentrations and are

normally kept in aqueous solution by virtue of incorporation into mixed micelles with the conjugated bile acids (1, 2). That the bile acids may actually be responsible for the biliary excretion of these lipids in the first place has been suggested by a variety of studies in several species (3-10). In general, it appears that increased excretion rates of bile acid are associated with increased absolute biliary concentrations and rates of excretion of lecithin and cholesterol but with reduced concentrations of the two lipids relative to the concentration of bile acid. In fact, the studies of Heath, Caple, and Redding (10) in the sheep and of Hardison and Francis (9) in the rat appear to have demonstrated absolute maximal rates of lipid excretion at the higher rates of bile acid excretion. Conversely, low rates of bile acid excretion are associated with the highest concentrations of lecithin and cholesterol relative to bile acid concentration, a condition most likely to predispose susceptible individuals to the risk of cholesterol precipitation (7, 8).

In an effort to delineate further the mechanisms of biliary lipid excretion the following study was undertaken in unanesthetized dogs.

METHODS

Female mongrel dogs (weights 17-20 kg) were prepared beforehand by cholecystectomy and installation of a Thomas cannula in the duodenum (11, 12). On the day of study the bile duct and one or both of the lateral saphenous veins were catheterized, initial samples of blood and bile were obtained, and i.v. infusions (e.g., saline, or solutions containing taurocholate, 14 C-labeled erythritol, or 14 C-labeled sucrose) were started (12). Approximately 30-60 min elapsed from the time of bile duct catheterization to the time the preparation was complete and the dog was upright in a sling. The anticholinergic drug pipenzolate methylbromide¹ was administered i.v. (0.5 mg/kg initially followed by 0.1 mg/kg every 20 min) in order to minimize fluctuations in bile flow (13).

¹ Pipenzolate methylbromide (Piptal) was generously supplied by Dr. Murray Finkelstein of Lakeside Laboratories, Milwaukee, Wis.

Received for publication 30 July 1971 and in revised form 12 October 1971.

TABLE I
Experimental Protocols

	A	B	C	D	E	F	G	H	I	J	K
I	T(8)	0	T(8)	T(8)	T(8)	T(8)	T(8)	T(8)	T(35)	0	0
II	T(35)	0	T(35)	T(8) + Se	T(8) + Se	T(8) + D(64)	T(8) + D(64)	T(8)	T(35) + Se	0	0
III	T(70)	T(8)	T(70)				T(8)	T(8)		Se	0
IV	D(32)		G(35)				T(8) + Se				
V	D(64)		G(70)								

Symbols: T(), G(), D() = infusions of sodium taurocholate, glycocholate, and dehydrocholate, respectively. Number shown in parentheses is approximate infusion rate in μ moles/min. Actual infusion rate was calculated in each case by calibration of pump speed and, in the case of taurocholate and glycocholate, measurement of bile acid concentration in infusion. Se, infusion of secretin at 4U/min; Sc, infusion of SC2644 at 11 μ moles/min; 0, no bile acid, secretin or SC2644 infusion.

Tracer infusions: 14 C-labeled erythritol was infused throughout each experiment except that in protocol G 14 C-labeled sucrose was infused and in protocol K no isotope was infused.

Timing: Bile collection periods are indicated by Roman numerals at left. In each case 30-60 minutes elapsed from the time of bile duct catheterization until the time tracer infusion was started and 90 min elapsed between initiation of tracer infusion and start of first bile collection, except that in protocol K (no tracer) 3 hr elapsed from the time of bile duct catheterization until the beginning of the first collection. Initial bile acid infusion (if any) was started at the same time as the tracer except in protocol I where it was started 60 min later. Actual bile collection periods lasted from 10 min (flows > 0.5 ml/min) to 30 min (flows < 0.09 ml/min). Whenever bile acid infusion rate was changed (A, B, C) 20-25 minutes elapsed before starting the next collection period. Whenever a new bile acid was infused (A and C) there was a 30 min hiatus without infusion before the new bile acid was started followed by another 25 min of equilibration on the new infusion. Whenever secretin (D) or SC2644 (E, G, I, J) were infused or when dehydrocholate was superimposed upon a taurocholate infusion (F, G) a 20 min equilibration period was allowed before starting a new bile collection. In protocols II and K and in the first two periods of B and J consecutive bile collections were obtained without interruption. Blood specimens for measurement of 14 C activity were obtained 2 min after starting each bile collection period except in protocol K.

11 different protocols were employed (Table I). Two typical experiments are shown in Table II.

Chromatography. Thin-layer chromatography was performed on the bile acid preparations used for infusion and on selected specimens of bile using silica gel G and the solvent systems described by Hofmann for conjugated and unconjugated bile acids (14). The bile specimens were prepared by shaking 0.2 ml of bile with 0.4 ml of chloroform-methanol (1:1) and 0.4 ml of water. The aqueous phase was subjected to chromatography for conjugated bile acids and the organic phase for unconjugated bile acids.

Analytical procedures. 14 C activity in plasma was estimated as described previously (12). Bile specimens were decolorized with sodium hypochlorite (Chlorox) as described (12) except that Cab-O-Sil was not used, and 50 μ l of 30% H_2O_2 was added to remove unreacted sodium hypochlorite before final dilution and addition of Bray's solution.

Biliary cholesterol concentration was measured by the method of Abell, Levy, Brodie, and Kendall (15). Biliary phospholipid was extracted in chloroform-methanol (2:1) and measured by the Bartlett procedure (16). Total bile acid concentration was determined by the hydroxysteroid dehydrogenase method of Talalay (17) as modified by Admirand and Small (18) using purified enzyme (STDHP—Worthington Biochemical Corp., Freehold, N. J.).

Materials. The sodium taurocholate employed for infusion (Mann Research Labs, Inc., New York) was an impure ox-bile preparation which contained 74% bile acid by weight and which was shown by thin-layer chromatography to contain a small amount of glycocholate, traces of taurine and glycine conjugates of dihydroxy bile acids, and traces of unconjugated cholic acid. The sodium glycocholate (ICN Nutritional Biochemicals Div., Irvine, Calif.) contained

61% bile acid by weight and although glycocholate was found to be the main constituent, it was heavily contaminated with glycine conjugated dihydroxy bile acids and with unconjugated cholic acid and contained traces of taurine conjugated di- and trihydroxy bile acids. Sodium dehydrocholate was obtained as a 20% solution (Decholin; Ames Co., Div. Miles Laboratories, Inc., Elkhart, Ind.) and appeared to be chromatographically pure. All bile acids were diluted to a concentration of approximately 20 mM in 5% dextrose and water and administered at the rates shown in Table I. Actual rates of infusion in each experiment were subsequently calculated from calibrated pump speed and infusion concentration. The latter was measured in the case of glycocholate and taurocholate and based upon the dilution of manufacturer's stated concentration in the case of dehydrocholate.

Secretin (GIH Laboratories, Stockholm) was diluted to a concentration of 10 U/ml in normal saline and administered at 4 U/min.

SC2644² (Fig. 1) was mixed with a small volume of water, dissolved by titration with 1 N NaOH to approximately pH 10 and diluted to a final concentration of 0.9%. Dextrose was added to a concentration of 5%. It was administered at 11 μ moles/min.

14 C-labeled erythritol and 14 C-labeled sucrose (both from Amersham/Searle Corp., Arlington Heights, Ill.) were dissolved and administered as described previously (12).

RESULTS

Relationships of biliary lipid excretions to taurocholate excretions. In the typical study shown in Table II so-

² SC2644 was generously provided by Donald L. Cook and G. D. Searle and Co., Skokie, Ill.

dium taurocholate was infused at three different rates. In each of the three states the measured excretion rate of bile acid was approximately equal to the rate of infusion, and the bile flow, erythritol clearance, lecithin excretion rate, and cholesterol excretion rate all increased with each increment in bile acid excretion rate. The absolute biliary concentrations of bile acid also increased and the relative concentrations of lecithin and cholesterol (expressed as per cent of the sum of molar concentrations of bile acid, cholesterol, and lecithin) declined. There was a fall in the molar ratio of lecithin-to-bile acid at the highest rate of infusion. The cholesterol-to-lecithin ratio showed no consistent trend and averaged 0.038.

In another study on the same dog (Table II) no bile acid was infused during the first two collection periods and then sodium taurocholate was infused at 8.8 $\mu\text{moles/min}$. During the first two periods the rate of endogenous bile acid excretion was low, as were the bile flow, erythritol clearance, and lipid excretion rates. Despite the low absolute biliary concentration of cholesterol its relative concentration was much higher than the relative concentration observed during taurocholate infusion. The cholesterol-to-lecithin ratio (average 0.109) was higher than the ratios (0.03 to 0.04) found at all higher rates of bile acid excretion. When sodium taurocholate was then infused at 8.8 $\mu\text{moles/min}$, the flow and composition of bile approached those observed in the first period of the previous study.

The lipid excretion data obtained from multiple studies on each dog during various rates of taurocholate infusion (or without bile acid infusion) are plotted in Figs. 2-6. The following characteristics were generally apparent: (a) Lecithin output was related to bile acid output by an ascending curve which was concave downward and appeared to pass through the origin. (b) The relationship of cholesterol output to bile acid output was qualitatively very similar to that described above except that the data obtained at the lowest rates of bile acid excretion in three of the dogs (Figs. 3, 5, and 6) suggested either that the true curve describing the relationship might have a positive intercept along the vertical axis or might have an abrupt change in shape near the

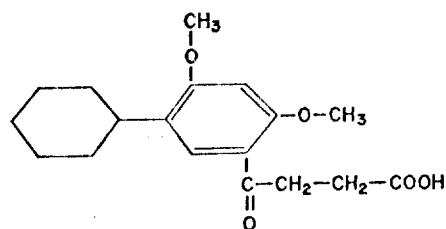


FIGURE 1 Structure of SC2644.

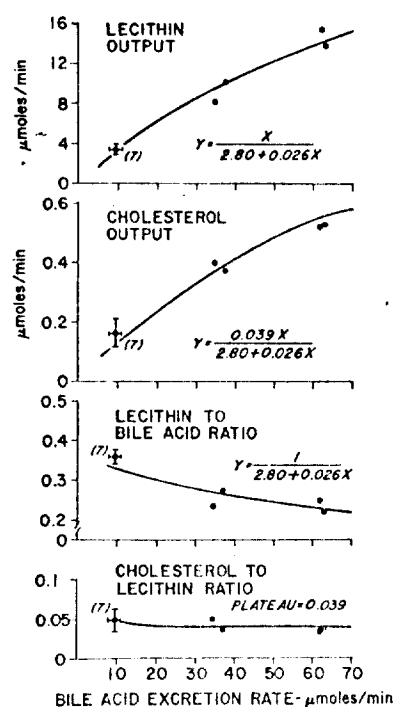


FIGURE 2 Effect of biliary excretion rate of bile acid upon outputs of lecithin and cholesterol and upon biliary molar concentration ratios of lecithin-to-bile acid and cholesterol-to-lecithin in dog Ha during sodium taurocholate infusion. The group of observations obtained during the lowest rate of infusion (circa 8 $\mu\text{moles/min}$) has been pooled, and the means, standard deviations, and number of observations (in parentheses) are shown for this group. At rates above 20 $\mu\text{moles/min}$ individual data points are shown. The lowest curve was fitted by eye and the other curves were fitted as described in the text.

origin. (c) Consistent with the relationship described in (a), the molar ratio of lecithin-to-bile acid declined gradually as bile acid output increased. (d) The cholesterol-to-lecithin ratio appeared to diminish rapidly toward a plateau of 0.031-0.042 as bile acid excretion rates rose to 8 $\mu\text{moles/min}$ or higher. This plateau was quite constant over a very wide range of bile acid excretion rates in each dog. At very low rates of bile acid excretion much higher cholesterol-to-lecithin ratios were observed except in one dog (dog Ad, Fig. 4) in which they were only slightly higher. No observations were made at the very low bile acid excretion rates in dog Ha (Fig. 2).

Curve fitting. Curves were fitted arbitrarily in the following manner: the curve for cholesterol-to-lecithin ratio (C/L)³ was fitted by eye and a numerical value

³ Abbreviations used in this paper: BA, bile acid; C, cholesterol; CMC, critical micellar concentration; L, lecithin; R, ratio.

TABLE II
Two Experiments Showing Effects of Various Bile Acid

Bile salt infusion	Infusion rate $\mu\text{mole}/\text{min}$	Time* of infusion		Time* of collection		Bile flow ml/min	Erythritol clearance ml/min	Bile acid Conc. mM
		Start	End	Start	End			
Taurocholate	7.4	-12	111	91	111	0.12	0.15	63.5
Taurocholate	30.9	111	150	135	150	0.29	0.30	92.3
Taurocholate	61.4	150	180	170	180	0.52	0.48	165.7
Dehydrocholate§	31.4	210	250	235	250	0.64	0.62	(3.5)†
Dehydrocholate§	62.3	250	280	270	280	0.77	0.85	(3.7)†
None	—	—	—	105	165	0.06	0.10	1.4
None	—	—	—	165	225	0.06	0.10	1.4
Taurocholate	8.8	226	291	261	291	0.12	0.16	1.7

* Zero time is arbitrarily defined as the time erythritol infusion was started, usually 30–60 min after catheterization of the common bile duct.

† Expressed as per cent of the sum of the molar concentrations of bile acid + lecithin + cholesterol.

§ During dehydrocholate infusion there was an appreciable biliary output of metabolites measurable by the 3-hydroxysteroid dehydrogenase method. These measurements, and the values derived therefrom, are given in parentheses since they obviously must underestimate the total output of dehydrocholate and its metabolites (and hence lead to overestimation of the moles per cent of lecithin and cholesterol and of the lecithin: bile acid ratios).

(R) for the ratio represented by the plateau was calculated as the average of all of the C/L values at bile acid excretion rates above 20 $\mu\text{moles}/\text{min}$. The curve relating the lecithin-to-bile acid ratio (L/BA) to the bile acid excretion rate was fitted by an equation of the form $y = 1/(A + Bx)$.⁴ These values of A and B were then used to plot the curve for lecithin excretion rate vs. bile acid excretion rate according to the equation $y = x/(A + Bx)$. Finally, the curve relating cholesterol output to bile acid output was plotted as $y = Rx/(A + Bx)$.

Except in the case of cholesterol output at the lowest rates of bile acid excretion (Figs. 3, 5, and 6) this procedure appeared to yield curves which fitted the data quite well. Other types of curves could probably have been fitted to the data just as well, but the particular hyperbolas were chosen for reasons which will be mentioned in the Discussion.

Relationship of biliary lipid excretion to glycocholate and dehydrocholate excretion. The infusion of sodium glycocholate (Table III) at two different rates (35 and 69 $\mu\text{moles}/\text{min}$) resulted in excretion rates of lecithin and cholesterol similar to those observed at comparable rates of sodium taurocholate infusion. In most instances

⁴ Based on least squares fit to a linear transform using a computer program available through General Electric Information Systems in BASIC language. In carrying out this fitting the mean coordinates of L/BA and BA output in the absence of bile acid infusion and the mean coordinates at low taurocholate infusion (circa 8 $\mu\text{moles}/\text{min}$) were each treated as a single data point.

the bile flows and erythritol clearances were somewhat higher with glycocholate than the taurocholate. As noted under Methods the "glycocholate" employed in these studies was impure and contained appreciable amounts of unconjugated cholic acid. During infusion of this material none of the unconjugated bile acids appeared as such in the bile, but appreciable quantities of taurocholate and some taurine conjugated dihydroxy bile acids were present. However, the major bile acids excreted were glycine conjugates in contrast to the dominant excretion of taurine conjugates when "taurocholate" was infused.

The effect of infusion of sodium dehydrocholate is illustrated by the study shown in Table II, and the data for five dogs are shown in Table III. The infusion of dehydrocholate at 32 and 64 $\mu\text{moles}/\text{min}$ was associated with appreciably higher rates of bile flow and with higher erythritol clearances than those observed during infusion of the natural bile salts at similar rates. The excretion rates of cholesterol and lecithin were very low at both rates of dehydrocholate infusion. The fact that they actually appeared to diminish during the more rapid infusion may not be significant, however, since this could have been due to progressive depletion of endogenous and previously administered natural bile acids.

Effect of secretin, SC2644, and dehydrocholate on biliary lipid excretion during constant infusion of sodium taurocholate. When sodium taurocholate was infused at a constant rate of approximately 7 $\mu\text{moles}/\text{min}$ (Table IV) the addition of secretin at 4 U/min caused an av-

Infusions on Bile Flow and Biliary Lipid Excretion (Dog Po)

Bile acid		Lecithin			Cholesterol			Lecithin: bile acid molar ratio	Cholesterol: lecithin molar ratio
Moles† per cent	Out- put	Conc.	Moles‡ per cent	Out- put	Conc.	Moles‡ per cent	Out- put		
	$\mu\text{mole}/\text{min}$	mM		$\mu\text{mole}/\text{min}$	mM		$\mu\text{mole}/\text{min}$		
73.4	7.6	22.1	25.5	2.6	0.94	1.1	0.11	0.35	0.043
72.5	28.8	36.5	26.6	10.6	1.18	0.9	0.34	0.37	0.032
80.0	55.0	25.5	19.3	13.3	1.01	0.8	0.52	0.24	0.040
(98.8)	(19.1)	0.7	(1.1)	0.4	0.09	(0.2)	0.06	(0.01)	0.138
(99.4)	(25.3)	0.3	(0.5)	0.2	0.08	(0.1)	0.06	(0.01)	0.256
71.1	0.9	5.7	26.3	0.3	0.57	2.6	0.03	0.37	0.100
71.9	0.8	4.5	25.1	0.3	0.53	3.0	0.03	0.35	0.118
74.8	8.9	25.2	24.6	2.9	0.70	0.7	0.08	0.33	0.028

erage increment of 0.24 ml/min in bile flow. In three of the four dogs there was only a small increment in erythritol clearance (average 0.05 ml/min) but in the fourth dog (Ad) there was an appreciable increment of 0.14 ml/min. (The unusual effect of secretin on the erythritol clearance in this single dog was confirmed in a nearly identical subsequent study and was borne out by demonstration of an identical effect of secretin on the mannitol clearance. This is the only dog in which

we have seen this phenomenon.) In none of the dogs was any change apparent in the excretion rates of bile salt, lecithin, or cholesterol.

The addition of SC2644 at 11 $\mu\text{moles}/\text{min}$ during a taurocholate infusion of 8 $\mu\text{moles}/\text{min}$ (Table V) caused marked increases in flow (average 0.41 ml/min), in erythritol clearance (mean increase 0.31 ml/min), and in sucrose clearance (mean increase 0.04 ml/min). In every instance there was a *decrease* in the excretion

TABLE III
Effect of Infusions of Taurocholate (T), Glycocholate (G), and Dehydrocholate (D) at Comparable Rates

Dog	Bile acid infusion rate			Bile flow			Erythritol clearance			Bile acid excretion rate			Lecithin excretion rate			Cholesterol excretion rate		
	T*	G	D	T*	G	D	T*	G	D	T*	G	D†	T*	G	D	T*	G	D
	$\mu\text{mole}/\text{min}$			ml/min			ml/min			$\mu\text{mole}/\text{min}$			$\mu\text{mole}/\text{min}$			$\mu\text{mole}/\text{min}$		
Ha	35.0	37.0	32.0	0.24	0.31	0.51	0.44	0.44	0.59	36.0	34.2	(20.3)	9.1	9.1	0.8	0.38	0.30	0.09
	69.1	70.3	66.0	0.40	0.66	0.60	0.55	0.77	0.78	62.4	65.5	(28.6)	14.6	13.7	0.3	0.52	0.42	0.07
La	32.1	34.0	32.7	0.31	0.37	0.49	0.34	0.42	0.51	32.5	33.5	(18.4)	8.1	9.1	0.7	0.27	0.29	0.04
	63.4	68.5	63.8	0.52	0.43	0.63	0.52	0.51	0.71	56.0	51.9	(28.5)	11.6	10.6	0.2	0.43	0.45	0.04
Ad	34.9	36.8	33.2	0.28	0.57	0.66	0.28	0.42	0.59	28.6	38.5	(18.8)	9.6	13.2	1.6	0.27	0.29	0.08
	68.7	72.5	65.3	0.48	0.63	0.93	0.44	0.61	0.80	55.1	57.1	(26.9)	13.6	14.8	0.5	0.49	0.47	0.06
Po	32.7	35.6	31.4	0.34	0.50	0.64	0.38	0.48	0.62	30.0	35.8	(19.1)	10.8	10.5	0.4	0.36	0.56	0.06
	62.8	66.1	62.3	0.56	0.70	0.77	0.55	0.74	0.85	56.9	56.7	(25.3)	14.1	12.7	0.2	0.51	0.42	0.06
Tu	42.7	38.4	36.6	0.40	0.37	0.65	0.39	0.36	0.65	38.6	28.3	(22.3)	10.7	7.5	0.6	0.44	0.31	0.10
	84.6	75.7	72.8	0.61	0.56	0.74	0.56	0.58	0.79	69.2	67.0	(33.5)	15.7	14.0	0.3	0.68	0.76	0.10

* All taurocholate data are averages of two separate studies.

† See footnote to Table II. Measured bile acid output values during dehydrocholate infusion include only the 3-hydroxysteroid dehydrogenase metabolites and are therefore shown in parentheses.

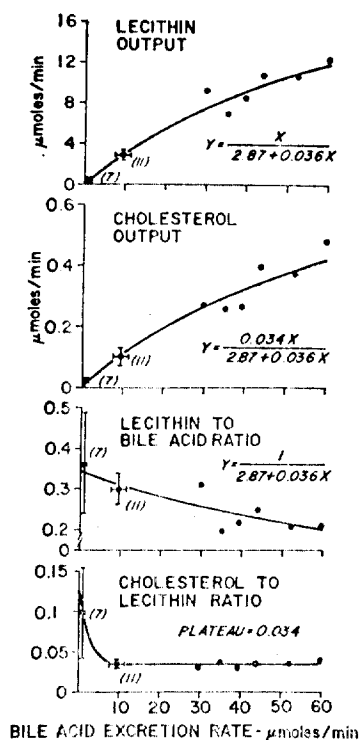


FIGURE 3 Effect of biliary excretion rate of bile acid upon outputs of lecithin and cholesterol and upon ratios of lecithin-to-bile acid and cholesterol-to-lecithin in dog La. Data were obtained in the absence of bile acid infusion and during sodium taurocholate infusion at various rates. Means, standard deviations, and number of observations (parentheses) are shown for the group of observations obtained in the absence of bile acid infusion and for the group obtained at the lowest rate of taurocholate infusion (circa 8 μ moles/min). Individual data points are plotted for all higher rates. The lowest curve was fitted by eye and the other curves were fitted as described in the text.

rates of both lecithin and cholesterol. The average decrement in lecithin excretion was 1.1 μ moles/min or 32%, and the decrement in cholesterol excretion was 0.03 μ moles/min or 34% when SC2644 was administered.

When sodium taurocholate was infused at 41 μ moles/min (Table VI) the addition of SC2644 at 11 μ moles/min caused increments in bile flow (average 0.30 ml/min) and erythritol clearance (average 0.31 ml/min) similar to those produced at the lower rate of taurocholate infusion, but caused no consistent changes in lecithin or cholesterol excretion. However, the basal rates of lipid excretion were high enough so that it would have been difficult to detect absolute decrements of the magnitude observed in the preceding experiments.

The addition of sodium dehydrocholate at 64 μ moles/min (Table V) during a taurocholate infusion of 8 μ moles/min caused increases in bile flow, erythritol clear-

TABLE IV
Effect of Secretin during Constant Infusion of Taurocholate at 7 μ moles/min

Dog	Condition	Flow	Erythritol clearance	Bile acid output	Lecithin output	Cholesterol output
		ml/min			μ mole/min	
Ha	Control	0.07	0.18	9.0	3.4	0.11
	Secretin	0.38	0.22	8.1	4.0	0.10
La	Control	0.11	0.16	10.5	3.6	0.18
	Secretin	0.28	0.20	9.7	3.4	0.11
Ad	Control	0.21	0.13	5.9	2.4	0.16
	Secretin	0.50	0.27	7.1	3.0	0.20
Po	Control	0.11	0.15	8.4	3.2	0.13
	Secretin	0.27	0.22	8.1	3.0	0.11

ance, and sucrose clearance averaging 0.55, 0.49, and 0.08 ml/min, respectively. Lecithin output decreased in each case (average decrement 1.1 μ moles/min or 32%) but cholesterol output showed little or no change.

Effect of SC2644 at low bile acid excretion rates. When SC2644 was administered approximately 4 hr after interruption of enterohepatic circulation to dogs receiving no bile acid infusion (Table VII) there was no obvious effect on total endogenous bile acid excretion

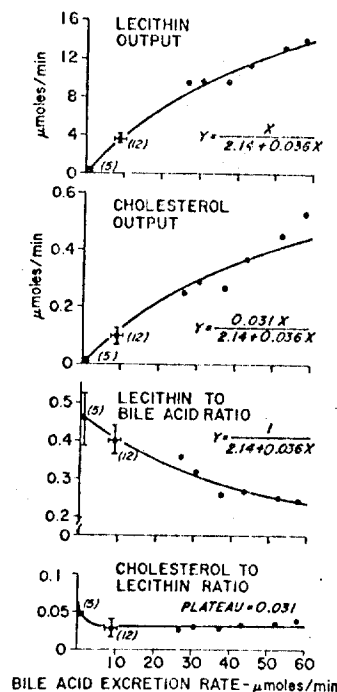


FIGURE 4 Effect of biliary excretion rate of bile acid upon outputs of lecithin and cholesterol and upon ratios of lecithin-to-bile acid and cholesterol-to-lecithin in dog Ad. See legend of Fig. 3 for details.

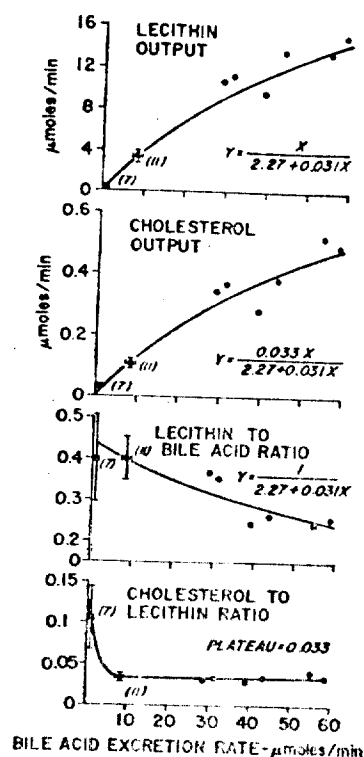


FIGURE 5 Effect of biliary excretion rate of bile acid upon outputs of lecithin and cholesterol and upon ratios of lecithin-to-bile acid and cholesterol-to-lecithin in dog Po. See legend of Fig. 3 for details.

rate but there was a diminution in the excretion rates of both lecithin and cholesterol in every dog. It should be noted that the effect of SC2644 on bile flow (average increase 0.28 ml/min) and on erythritol clearance (average increase 0.25 ml/min) were very similar to those observed during bile acid infusion of 8 (Table V) and 41 (Table VI) μ moles/min.

Observations on possible effects of time on bile composition. Three consecutive 30 min collections of bile during constant taurocholate infusion at 8 μ moles/min (Table VIII) showed remarkably constant outputs and concentration ratios. During total interruption of enterohepatic circulation (also in Table VIII) consecutive 10 min collections showed considerably greater variation. However, in two of three dogs there was no consistent trend with the passage of time. In one experiment on the second dog (La) there was a sixfold decrease in endogenous bile acid excretion rate and the changes in cholesterol and lecithin outputs and concentration ratios were consistent with those predicted during deliberate manipulation of bile acid output (Fig. 3).

The trend in this experiment could therefore be attributed to the effects of delayed depletion of the enterohepatic circulating bile acid pool. The studies shown in Table VIII thus provide no evidence for alterations in bile composition attributable to temporal changes in hepatic lipid metabolism during the relatively brief total times required to execute any of the experiments shown in the preceding tables.

Initial "common duct" bile specimens. At the beginning of each study 3-6 ml of bile drained from the common bile duct within the first few minutes after catheterization. Most of this bile had probably been stored in the bile ducts for some time before catheterization and, as noted in earlier studies (19), it tended to have a high concentration of bile acid. The average composition of all "common duct" bile specimens is shown in Table IX. Although the absolute concentrations of bile acids and lipids were very high in most instances the ratios of these constituents were well within the range encountered in flowing hepatic bile at intermediate rates of bile acid excretion (Figs. 2-6).

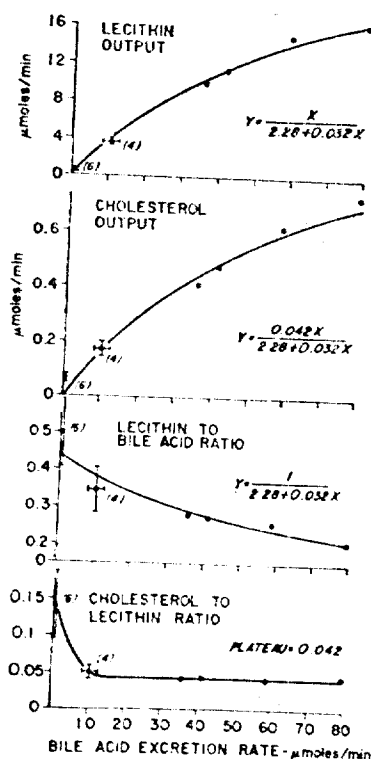


FIGURE 6 Effect of biliary excretion rate of bile acid upon outputs of lecithin and cholesterol and upon ratios of lecithin-to-bile acid and cholesterol-to-lecithin in dog Tu. See legend of Fig. 3 for details.

TABLE V
Effect of Dehydrocholate and SC2644 during Constant Sodium Taurocholate Infusion at 8 μ moles/min*

Dog	Condition	Bile flow	Erythritol clearance	Sucrose clearance	Bile acid output†	Lecithin output‡	Cholesterol output	Suc:eryth clearance ratio	Estimated $\sigma_{sucrose}$ §
		ml/min			μ mole/min				
Ha*	Control	0.06	0.13	—	7.2	2.8	0.08	—	—
	SC2644	0.56	0.44	—	6.9	1.3	0.04	—	—
La	Control	0.09	0.19	0.029	10.8	3.0	0.09	0.15	
	Dehydrocholate	0.57	0.68	0.079	—	1.5	0.06	0.12	0.88
	Control	0.09	0.14	0.028	8.0	2.6	0.08	0.20	
	SC2644	0.46	0.40	0.063	8.5	1.8	0.06	0.16	0.84
Ad	Control	0.16	0.18	0.041	10.6	4.4	0.12	0.24	
	Dehydrocholate	0.74	0.58	0.150	—	3.3	0.12	0.26	0.74
	Control	0.15	0.19	0.060	10.6	4.0	0.10	0.32	
	SC2644	0.42	0.46	0.098	11.1	2.8	0.08	0.21	0.79
Po	Control	0.13	0.14	0.018	7.2	2.8	0.10	0.34	
	Dehydrocholate	0.72	0.72	0.131	—	1.8	0.10	0.18	0.82
	Control	0.17	0.14	0.018	9.9	4.1	0.12	0.34	
	SC2644	0.52	0.53	0.096	8.9	1.7	0.07	0.18	0.82

* With the exception of dog Ha each bile flow and output value is the average of two determinations made on different days. The erythritol clearance was estimated on one of the two days and the sucrose clearance on the other. All control values shown were those obtained immediately before the administration of the choleretic agent indicated in the row that follows. Sodium dehydrocholate was administered at 64 μ moles/min and SC2644 at 11 μ moles/min.

† Total bile acid outputs are not reported in the dehydrocholate studies because chemical measurement of concentration only included 3-hydroxysteroid metabolites (see footnote to Table II). Since taurocholate was infused throughout these studies the output of taurocholate is assumed to have been at least equal to infusion rate.

§ Reflection coefficient for sucrose ($\sigma_{sucrose}$) was estimated as 1 minus the sucrose:erythritol clearance ratio (see text).

DISCUSSION

Dependence of biliary lipid excretion upon excretion of micelle-forming bile acids

The biliary excretion rates of lecithin and of cholesterol in these dogs, as in the isolated dog liver (5) and in all other species studied previously (6-8, 10), appeared to be intimately dependent upon the biliary excretion rate of the bile acids. That this phenomenon is

attributable exclusively to the ability of the bile acids to form micelles and not to the effect of bile acids on bile flow is demonstrated by the fact that three other

TABLE VII
Effect of SC2644 in the Absence of Bile Acid Infusion

Dog	Condition	Bile flow	Erythritol clearance	Bile acid output	Lecithin output	Cholesterol output
		ml/min		μ mole/min		
La	Control	0.04	0.15	1.03	0.30	0.026
	Control	0.06	0.11	0.37	0.08	0.016
	SC2644*	0.35	0.28	0.37	0.02	0.004
Ad	Control	0.06	0.07	0.92	0.34	0.018
	Control	0.07	0.08	0.62	0.28	0.015
	SC2644*	0.29	0.24	0.92	0.12	0.011
Po	Control	0.07	0.11	1.44	0.81	0.037
	Control	0.06	0.09	1.25	0.58	0.030
	SC2644*	0.41	0.39	1.03	0.15	0.004
Tu	Control	0.09	0.12	1.43	0.63	0.059
	Control	0.16	0.14	1.14	0.47	0.054
	SC2644*	0.43	0.50	.94	0.11	0.014

* Infused at 11 μ moles/min after two consecutive control periods during which the enterohepatic pool became depleted. Approximately 4 hr had elapsed from the time bile duct was catheterized to the time SC2644 was started (protocol J).

TABLE VI
Effect of SC2644 during Constant Sodium Taurocholate Infusion at 41 μ moles/min*

Dog	Condition	Bile flow	Erythritol clearance	Bile acid output	Lecithin output	Cholesterol output
		ml/min		μ mole/min		
La	Control	0.30	0.34	41.7	9.8	0.33
	SC2644†	0.64	0.67	43.1	7.0	0.20
Ad	Control	0.38	0.40	40.2	10.5	0.32
	SC2644†	0.61	0.68	44.3	11.5	0.30
Po	Control	0.40	0.41	41.4	10.5	0.33
	SC2644†	0.73	0.72	47.4	9.4	0.27

* Each number is the average of two determinations made on different days.

† SC2644 was infused at 11 μ moles/min.

potent choleric agents failed to enhance (or actually reduced) lipid excretion.

(a) *Secretin*. Secretin, which acts primarily by promoting fluid secretion by the bile ducts or ductules (20-22) had no effect on lipid excretion (Table IV).

(b) *Dehydrocholate*. Dehydrocholate infusion failed to cause any increase in lipid excretion rates (Tables II, III, and V). This is similar to the findings of Hardison and Francis (9) in rats. The choleric action of dehydrocholate, like that of the natural bile acids, can be attributed to an increase in the rate of canalicular fluid production as indicated by the increment in erythritol clearance. Dehydrocholate differs from the natural bile acids in that it does not form micelles. However, dehydrocholate is converted to a variety of metabolites before biliary excretion (23). In the present studies, for example, the excretion rate of 3-hydroxylated metabolites (as measured by the hydroxysteroid dehydrogenase reaction) was equal to about one-half of the infusion rate of dehydrocholate (Tables II, III, and V). Since most of the dehydrocholate metabolites, like the parent compound, do not form micelles (23), it is reasonable to assume that lack of micelle formation is responsible for the failure of dehydrocholate to enhance lipid excretion. However, interpretation is complicated by the fact that a small fraction of administered dehydrocholate is actually converted to cholic acid (23). It is possible that if this did not occur there might have been more obvious decrements in lipid excretion such as those observed with SC2644.

(c) *SC2644*. This potent choleric compound also failed to enhance lipid excretion rates (Tables V, VI, and VII). The mechanism responsible for its choleric effect is unknown, but it is germane to this discussion to examine the evidence regarding its behavior and site of action. Like dehydrocholate, SC2644 apparently caused increased canalicular fluid output as indicated by the increase in erythritol clearance. On either a molar or weight basis it is obviously a far more potent choleric agent than dehydrocholate in the dog and is more potent than any of the chemically related organic acids studied by Gunter, Kim, Magee, Ralston, and Ivy (24). In fact even on the assumption that it was all secreted into the bile canaliculi, the response to the small dose employed was too great and too prolonged (persisting in these studies for at least 3 hr after cessation of about 30 min of infusion at 11 μ moles/min) to be readily explained on the basis of an osmotic effect of SC2644 per se. Insofar as the quantity (1 - sucrose clearance/erythritol clearance) may be used as a rough estimate of the reflection coefficient for sucrose (σ_{sucrose}) at high flows (12, 25) it would appear that this parameter of passive membrane permeability was identical whether choleresis was produced by SC2644 or dehydrocholate (Table V). Moreover, a given dose of SC2644 appeared to produce

a nearly identical increment in bile flow and erythritol clearance at three widely different rates of basal flow and bile acid secretion rate (compare Tables V, VI, and VII). Stimulation of "bile acid independent" (12, 26, 27) inorganic solute and water transport into the canaliculi would thus appear to be the most logical tentative explanation for the effect of this agent. Therefore, bile acid secreted into the canalicular lumen would be diluted as a result of SC2644 administration.

Evidence that lipid entry depends upon the micellar state of bile acids in the canaliculi

Bile acids might affect the output of lipids by two possible mechanisms. First, lecithin and cholesterol might be "entrained" at some stage during bile acid transport before actual entry into the canaliculi. Or second, the presence of bile acid micelles within the canalicular lumen might serve to trap lipids which entered via the membrane surrounding the canalicular lumen. The latter possibility is supported by the fact that SC2644 actually *reduced* lipid output during the course of a constant slow infusion of taurocholate (Table V) and that SC2644 caused a marked reduction in lecithin and cholesterol excretion when administered during the course of the very low bile acid excretion rates that accompanied interrupted enterohepatic circulation (Table VII). It is postulated that canalicular fluid produced in response to SC2644 diluted the intracanicular bile acids toward their critical micellar concentration so that a larger proportion of the total bile acid was in the dissociated state and a smaller proportion was available for solubilization of lipids. In other words, the present results are consistent with the view that the rate of biliary excretion of lipids is a function of the solubilizing capacity of the bile acids within the canalicular lumen rather than the total bile acid excretion rate.

Quantitative relationships between lecithin excretion and bile acid excretion

The present studies do not indicate whether biliary lipids are originally derived directly from the canalicular membranes as has been suggested by Small (28) or whether their origin is intrahepatic. In either case it is possible to conceive of a model based on passive lipid movement which is consistent with most of the present observations. The solubilities of lecithin and cholesterol in water are exceedingly small. Nonetheless it can be assumed that individual lipid molecules will escape transiently into the canalicular aqueous phase from either the membrane or the bile acid micelles and then return to either at random. The net movement of a lipid species between membrane phase and micellar phase should thus be a function of the relative tendency

TABLE VIII
Sequential Bile Collections with and without

Dog	Taurocholate infusion rate	Bile flow			Erythritol clearance			Bile acid output		
		1	2	3	1	2	3	1	2	3
	$\mu\text{mole/min}$		ml/min			ml/min			$\mu\text{mole/min}$	
Ha	8.1	0.10	0.11	0.11	0.16	0.16	0.14	10.3	9.2	8.2
La	7.9	0.10	0.12	0.12	0.12	0.16	0.13	8.4	9.0	9.3
	0	0.02	0.04	—	0.06	0.07	—	0.84	0.77	—
	0	0.03	0.02	0.03	—	—	—	2.59	1.13	0.44
Ad	7.8	0.12	0.15	0.14	0.16	0.19	0.17	8.0	9.8	8.2
	0	0.14	0.10	—	0.10	0.08	—	1.04	0.61	—
	0	0.06	0.05	0.04	—	—	—	1.33	1.17	1.14
Po	7.6	0.13	0.18	0.14	0.18	0.22	0.22	8.0	9.6	8.0
	0	0.06	0.06	—	0.10	0.10	—	0.85	0.80	—
	0	0.06	0.06	0.05	—	—	—	0.69	1.08	0.48

* Protocols H, B, and K (Table I).

of lipid to escape from either phase. In the case of lecithin one could assume, for example, that the escaping tendency from membrane to lumen had some fixed value P_1 and that the escaping tendency from the micelles P_2 was related to the concentration of lecithin within the micelles. The latter assumption is based upon the finding of Mysels (29) that the concentration of lipid soluble dye in the aqueous phase of a micellar solution is directly proportional to the ratio of dye to solubilizer in the micellar phase. The relationships between bile acid output and lecithin output could then be predicted as follows: P_1 , escaping tendency of lecithin from membrane; P_2 , escaping tendency of lecithin from micelles; L , biliary lecithin concentration; B , biliary micellar bile acid concentration; J_L , net lecithin excretion rate into bile; J_B , net bile acid excretion rate into bile; K_1 , K_2 , arbitrary constants (K_1 is analogous to the partition constant between aqueous and micellar phase described by Mysels, and K_2 is analogous to a diffusion constant across the region separating membrane from micelles).

Assuming that escaping tendency from micelles is proportional to the ratio of lecithin-to-bile acid in the micelles:

$$P_2 = K_1(L/B).$$

($B \approx$ total bile acid concentration where the latter greatly exceeds the critical micellar concentration.)

$$L/B = J_L/J_B$$

$$\therefore P_2 = K_1(J_L/J_B).$$

Assuming net lecithin flux is proportional to the differ-

ence between escaping tendencies:

$$J_L = K_2(P_1 - P_2)$$

$$J_L = K_2(P_1 - K_1(J_L/J_B)).$$

Rearranging:

$$J_L = \frac{J_B}{K_1/P_1 + (1/K_2P_1)J_B} \quad (1)$$

also

$$J_L/J_B = L/B = \frac{1}{K_1/P_1 + (1/K_2P_1)J_B} \quad (2)$$

There are a number of obvious oversimplifications in this approach, but the model predicts a hyperbolic ascending relationship between lecithin excretion and bile acid excretion and a declining value of lecithin-to-bile acid ratio at increasing rates of bile acid excretion. On this basis hyperbolic functions of the form predicted by equation 2 were fitted to the data for lecithin-to-bile acid ratio, and the constants derived from the fitting procedure (K_1/P_1 and $1/K_2P_1$) were then introduced into equation 1 to obtain the curves for lecithin excretion rates. It is evident that a reasonable fit was obtained in every case (Figs. 2-6) so that at least the data were consistent with the model described. It should be noted that Heath et al. (10) and Hardison and Francis (9) observed apparent "plateaus" or maximal lecithin excretion rates under conditions of bile acid loading in sheep and rats, respectively. Such a phenomenon would be predicted from equation 1 even though plateaus were not actually achieved in

Constant Infusions of Sodium Taurocholate*

Lecithin output			Cholesterol output			Lecithin: bile acid molar ratio			Cholesterol: lecithin molar ratio		
1	2	3	1	2	3	1	2	3	1	2	3
$\mu\text{mole/min}$			$\mu\text{mole/min}$								
3.5	3.5	2.8	0.21	0.22	0.19	0.34	0.38	0.34	0.060	0.063	0.068
2.3	2.6	2.5	0.09	0.10	0.10	0.28	0.29	0.27	0.038	0.038	0.038
0.29	0.47	—	0.024	0.033	—	0.35	0.61	—	0.084	0.070	—
0.72	0.36	0.21	0.028	0.019	0.029	0.28	0.32	0.48	0.038	0.054	0.140
3.1	3.8	3.1	0.08	0.09	0.09	0.39	0.39	0.38	0.026	0.024	0.020
0.42	0.32	—	0.017	0.014	—	0.39	0.54	—	0.042	0.042	—
0.70	0.52	0.42	0.024	0.025	0.022	0.53	0.45	0.37	0.034	0.041	0.052
3.3	3.9	3.4	0.09	0.12	0.10	0.41	0.41	0.43	0.029	0.031	0.028
0.31	0.28	—	0.031	0.033	—	0.37	0.35	—	0.100	0.118	—
0.23	0.20	0.25	0.033	0.026	0.034	0.33	0.18	0.52	0.148	0.133	0.136

the dog. In equation 1, as $J_b \rightarrow \infty$ the predicted value of J_b would be K_2P_1 or roughly 30 $\mu\text{moles/min}$ in the present animals. However, this value would actually be unattainable because of the limitation imposed by a transport maximum for bile acids of the order of 150 $\mu\text{moles/min}$ (30) in dogs of this size.

Relationship of cholesterol excretion to bile acid and lecithin excretion, evidence for cholesterol-lecithin coupling

The output of cholesterol, like that of lecithin appears to depend upon the bile acid excretion rate (Figs. 2-6).

However, the fact that the cholesterol-to-lecithin ratio was approximately constant over a wide range of bile acid excretion rates and a wide range of outputs of cholesterol and lecithin is consistent with only the following two possibilities: (a) That there is a fortuitous relationship between the factors which determine the independent rates of movement of the two lipids. For example, if the rate of cholesterol excretion (J_c) were described by the following equation (analogous to equation 1):

$$J_c = \frac{J_b}{K'_1/P'_1 + (1/K'_2P'_1)J_b}$$

TABLE IX
Composition of Initial Bile Drained from Common Duct

Dog		Bile acid	Lecithin	Cholesterol	L:BA ratio	C/L ratio	Moles per cent		
							BA	L	C
			$\mu\text{mole/liter}$						
Ha (N = 5)	Mean	244.6	86.8	2.90	0.36	0.035	73.2	25.9	0.86
	SD	24.2	18.7	0.69	0.10	0.010	4.8	4.9	0.15
La (N = 12)	Mean	193.3	51.9	2.03	0.27	0.039	77.9	21.2	0.83
	SD	39.9	8.0	0.61	0.04	0.008	2.6	2.5	0.22
Ad (N = 11)	Mean	95.6	26.9	0.94	0.29	0.036	77.2	22.1	0.78
	SD	21.5	5.5	0.18	0.06	0.010	3.3	3.3	0.19
Po (N = 12)	Mean	102.3	35.2	1.13	0.35	0.032	73.8	25.4	0.82
	SD	13.0	6.2	0.26	0.05	0.006	2.8	2.7	0.18
Tu (N = 6)	Mean	119.7	46.2	1.84	0.39	0.041	71.6	27.4	1.09
	SD	26.2	13.2	0.56	0.10	0.009	4.8	4.8	0.18

then the ratio of cholesterol-to-lecithin would be constant for all values of J_B only if $K_1K_2 = K'_1K'_2$. (b) That there is an association or coupling between the two lipid species which determines their relative rates of movement into the bile canaliculi. For example, the association between cholesterol and lecithin in their region of origin (e.g., in canalicular membrane) might be such that an average of one molecule of cholesterol would accompany every 25–30 molecules of lecithin into the canaliculi. In this view cholesterol and lecithin might make the transition from membrane to intracanalicular micelle in the form of dimers or other complexes. Since the constants K_1 and K_2 (or K'_1 and K'_2) describe such different properties of the molecule, the relationships required by the first possibility appear to be unlikely and the second possibility—that of coupling between lecithin and cholesterol movement—appears to provide the more attractive explanation for the constancy of the cholesterol-to-lecithin ratio. Because of this viewpoint the curves which were fitted to the cholesterol output vs. bile acid output data (Figs. 2–6) were calculated simply by multiplying the equation for the lecithin output vs. bile acid output curve by a fixed value equal to the value of the cholesterol-to-lecithin “plateau.”

High cholesterol-to-lecithin ratios at very low bile acid excretion rates, evidence for cholesterol entry independent of lecithin

A separate mechanism must be invoked to explain the high cholesterol-to-lecithin ratios observed during total interruption of the enterohepatic circulation. It is possible that it is caused by a slow but continuous secretion of cholesterol independent of bile acid output. It is also possible that there is slow passive movement of cholesterol into the canaliculi *in response* to bile acid secretion but independent of the coupled lecithin-cholesterol movement suggested in the preceding paragraph. The fact that cholesterol excretion dropped markedly when SC2644 was administered in the absence of bile acid infusion (Table VII) appears to support the latter possibility. Although SC2644 did not consistently alter total bile acid excretion rate the marked enhancement of canalicular fluid production (indicated by an average increment in erythritol clearance of 0.25 ml/min) can be estimated to have reduced the canalicular bile acid concentration to the range of 1.5–2.5 mmoles/liter. The critical micellar concentration (CMC) of bile acids in whole bile has not been measured in the dog, but these values are close to the range of the CMC reported by Tamesue and Juniper for whole human bile (31) and it may be assumed that an appreciable fraction of the bile acid was in the dissociated state. The resultant reduction in cholesterol ex-

cretion suggests that the entry of cholesterol even at very low rates of bile acid output is still dependent upon the availability of bile acid micelles and is not an independent transport process.

The following modification of the model proposed for lecithin excretion would suffice to explain the data describing cholesterol output and cholesterol-to-lecithin ratios. If it is assumed that entry of cholesterol independent of lecithin obeys the same rules as those described in the derivation of equation 1 then total cholesterol output would be:

$$J_c = \frac{J_B}{K'_1/P'_1 + (1/K'_2P'_1)J_B} + \frac{RJ_B}{K_1/P_1 + (1/K_2P_1)J_B}$$

where P_1 , K_1 , and K_2 are the constants described previously for lecithin, P'_1 , K'_1 , and K'_2 are the analogous constants for cholesterol and R is the coupling ratio which describes the average number of moles of cholesterol which accompany each mole of lecithin.

The cholesterol-to-lecithin ratio would then be:

$$C/L = \frac{K_1/P_1 + (1/K_2P_1)J_B}{K'_1/P'_1 + (1/K'_2P'_1)J_B} + R \quad (5)$$

Equations 4 and 5 have been plotted in the appropriate panels of Fig. 7 after substituting the following values: $K_1P_1 = 2.5$, $1/K_2P_1 = 0.03$, $R = 0.04$, $K'_1P'_1 = 5$, $1/K'_2P'_1 = 30$. The first three are average values based upon the results of the curve fitting already described. The last two are arbitrary choices although it is not unreasonable to suppose that the tendency for cholesterol to escape from the membrane might be much less than that for lecithin (i.e., $P'_1 \ll P_1$) from which it would be likely that $1/K'_2P'_1 \gg 1/K_2P_1$, and that K'_1 , the constant included in the escaping tendency of cholesterol from micelles, might also be low enough so that K'_1/P'_1 would be of the same order of magnitude as K_1/P_1 .

It may be seen from Fig. 7, on which equations 1 and 2 have also been plotted, that the hypothetical model, including the provision for a dual mechanism of cholesterol excretion, now provides an adequate description of the entire range of data shown in Figs. 2–6.

Possible role of changes in lipid synthetic rates

Previous studies have demonstrated a positive effect of bile acid on the synthetic rates of hepatic lecithin (32, 33). In the present acute studies, however, there were no consistent changes in biliary lipid excretion with time (Table VIII) during either totally inter-

rupted enterohepatic circulation or constant bile acid infusion. Thus it appears improbable that there was sufficient time for changes in the rates of lipid synthesis to contribute to any of the relationships observed.

"History" of bile found in the common bile duct deduced from its composition

In the cholecystectomized dog the common bile duct provides a reservoir which usually contains 4-6 ml of bile. In the fasting state the bile first aspirated after catheterization (so called "common duct bile") is often indistinguishable in composition from highly concentrated gallbladder bile (19). This is consistent with the known reabsorptive function of the ducts (12). Thus in the present studies, the absolute concentrations of bile acids, lecithin, and cholesterol were often very high in common duct bile (Table IX). The relative concentrations of these constituents, when compared with the other data obtained, provide some indication of the average bile acid excretion rates prevailing at the time the "common duct" specimens were produced by the liver. For example, the mean lecithin-to-bile acid ratios of 0.27 to 0.39 would suggest that this bile was produced at average bile acid excretion rates of roughly 10-30 μ moles/min (based on Figs. 2-6). Thus the bulk of the lipid in this bile was probably excreted during periods of appreciable enterohepatic bile acid circulation. Analysis of a single sample of common duct bile (or, in the intact animal, of gallbladder bile) obviously provides no clue to the range of composition of hepatic bile which may be secreted at various times and under various circumstances in the same animal.

Implications

It is well recognized that the dog is not susceptible to cholesterol gallstone formation. All of the present data lie well within the solubility zone for cholesterol when plotted on the triangular coordinates described by Admirand and Small (18). Nevertheless, an unanesthetized dog may be studied repeatedly and provides a reproducible means of examining the relationship between biliary bile acid and lipid excretion. Our evidence suggests that the micellar state of the bile acids within the canaliculi controls lecithin and cholesterol excretion, and is therefore consistent with the view that these lipids enter via the membranes immediately surrounding the canaliculi. Small (28) has suggested that the biliary lipids may actually represent solubilized membrane constituents, and that the formation of bile which is supersaturated with respect to cholesterol may occur by incorporation of associated cholesterol and lecithin into micelles in a high cholesterol-to-lecithin ratio which is ultimately inconsistent with micellar stability. This possibility is strongly supported by our

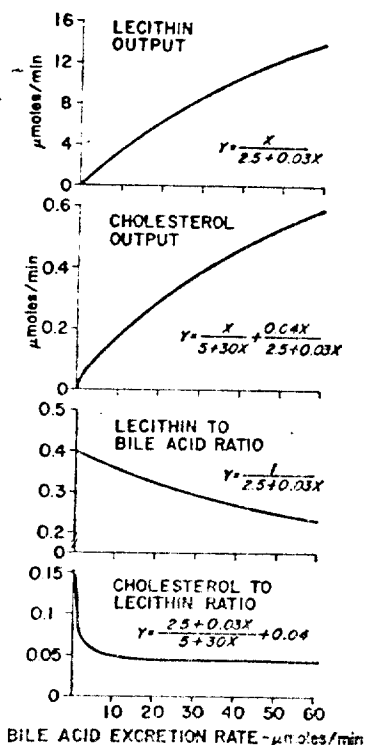


FIGURE 7 Hypothetical curves describing effect of biliary excretion rate of bile acid upon outputs of lecithin and cholesterol and upon ratios of lecithin-to-bile acid and cholesterol-to-lecithin. The derivation of these curves was based on the assumptions that lecithin moves passively from cell membranes to intercanicular micelles, that transport of cholesterol is coupled to lecithin transport, and that there is also a small amount of independent passive transport of cholesterol from membranes to micelles (see text).

evidence for cholesterol-lecithin coupling. Although obviously no threat to the dog, such coupling could, in other species and under other circumstances, lead to the formation of unstable micelles.

The finding that a very small amount of cholesterol may enter the bile independent of lecithin is interesting, but this process also appears to depend upon the availability of bile acid micelles within the canaliculi. It seems highly unlikely that cholesterol entering in this fashion could ever lead to the production of a bile which was supersaturated with respect to cholesterol.

If biliary lipid excretion is the result of solubilization of canalicular membrane it may perhaps be regarded as an unnecessary (and sometimes unfortunate) by-product of the biliary secretion of powerful detergent compounds necessary for digestion. Our results suggest that the avidity with which the membrane retains its lipid constituents could be the major determinant of biliary lipid composition.

Note added in proof. Since the preparation of this manuscript Hardison and Apter (Hardison, W. G. M., and J. T. Apter. 1972. Micellar theory of biliary cholesterol excretion. *Amer. J. Physiol.* 222: 61) have reported biliary lipid excretion patterns in the rat which are similar in many respects to those described in the present paper and which expand upon the results published in the earlier abstract of Hardison and Francis (9).

ACKNOWLEDGMENTS

The authors are grateful to Mrs. Helene Curtis and Mrs. Nina Pollock for their valuable technical assistance.

This work was supported by U. S. Public Health Service Grant AM 13097.

REFERENCES

- Isaksson, B. 1954. On dissolving power of lecithin and bile salt for cholesterol in human bladder bile. *Acta Soc. Med. Upsal.* 59: 296.
- Bourges, M., D. M. Small, and D. G. Dervichian. 1967. Biophysics of lipid associations. III. The quaternary systems lecithin-bile salt-cholesterol-water. *Biochim. Biophys. Acta.* 144: 189.
- Kay, R. E., and C. Entenman. 1961. Stimulation of taurocholic acid synthesis and biliary excretion of lipids. *Amer. J. Physiol.* 200: 855.
- Entenman, C., R. J. Holloway, M. L. Albright, and G. F. Leong. 1968. Bile acids and lipid metabolism. I. Stimulation of bile lipid excretion by various bile acids. *Proc. Soc. Exp. Biol. Med.* 127: 1003.
- Swell, L., C. C. Bell, Jr., and C. Entenman. 1968. Bile acids and lipid metabolism. III. Influence of bile acids on phospholipid in liver and bile of the isolated perfused dog liver. *Biochim. Biophys. Acta.* 164: 278.
- Swell, L., C. Entenman, G. F. Leong, and R. J. Holloway. 1968. Bile acids and lipid metabolism. IV. Influence of bile acids on biliary and liver organelle phospholipids and cholesterol. *Amer. J. Physiol.* 215: 1390.
- Dowling, R. H., and D. M. Small. 1968. The effect of controlled interruptions of the enterohepatic circulation on the composition of bile in the rhesus monkey. *J. Clin. Invest.* 47: 26a.
- Nilsson, S., and T. Scherstén. 1969. Importance of bile acids for phospholipid secretion into human bile. *Gastroenterology.* 57: 525.
- Hardison, W. G. M., and T. I. Francis. 1969. The mechanism of cholesterol and phospholipid excretion in bile. *Gastroenterology.* 56: 1164.
- Heath, T., I. W. Caple, and P. M. Redding. 1970. Effect of the enterohepatic circulation of bile salts on the flow of bile and its content of bile salts and lipids in sheep. *Quart. J. Exp. Physiol.* 55: 93.
- Thomas, J. E. 1941. An improved cannula for gastric and intestinal fistulas. *Proc. Soc. Exp. Biol. Med.* 46: 260.
- Wheeler, H. O., E. D. Ross, and S. E. Bradley. 1968. Canalicular bile production in dogs. *Amer. J. Physiol.* 214: 866.
- Preisig, R., H. L. Cooper, and H. O. Wheeler. 1962. The relationship between taurocholate secretion rate and bile production in the unanesthetized dog during cholinergic blockade and during secretin administration. *J. Clin. Invest.* 41: 1152.
- Hofmann, A. F. 1962. Thin-layer adsorption chromatography of free and conjugated bile acids on silicic acid. *J. Lipid Res.* 3: 127.
- Abell, L. L., B. B. Levy, B. B. Brodie, and F. E. Kendall. 1952. A simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. *J. Biol. Chem.* 195: 357.
- Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* 234: 466.
- Talalay, P. 1960. Enzymic analysis of steroid hormones. *Methods Biochem. Anal.* 8: 119.
- Admirand, W. H., and D. M. Small. 1968. The physicochemical basis of cholesterol gallstone formation in man. *J. Clin. Invest.* 47: 1043.
- Wheeler, H. O., and O. L. Ramos. 1960. Determinants of the flow and composition of bile in the unanesthetized dog during constant infusion of sodium taurocholate. *J. Clin. Invest.* 39: 161.
- Wheeler, H. O., and P. L. Mancusi-Ungaro. 1966. Role of bile ducts during secretin choleresis in dogs. *Amer. J. Physiol.* 210: 1153.
- O'Maille, E. R. L., T. G. Richards, and A. H. Short. 1966. Factors determining the maximal rate of organic anion secretion by the liver and further evidence on the hepatic site of action of the hormone secretin. *J. Physiol. (London).* 186: 424.
- Forker, E. L. 1967. Two sites of bile formation as determined by mannitol and erythritol clearance in the guinea pig. *J. Clin. Invest.* 46: 1189.
- Soloway, R. D., A. F. Hofmann, P. J. Thomas, and L. J. Schoenfeld. 1971. Dehydrocholic acid: hepatic metabolism and effect of biliary secretion in man. *Gastroenterology.* 60: 754.
- Gunter, M. J., K. S. Kim, D. F. Magee, H. Ralston, and A. C. Ivy. 1950. The choleretic potencies of some synthetic compounds. *J. Pharmacol. Exp. Ther.* 99: 465.
- Forker, E. L. 1969. The effect of estrogen on bile formation in the rat. *J. Clin. Invest.* 48: 654.
- Boyer, J. L., and G. Klatskin. 1970. Canalicular bile flow and bile secretory pressure. Evidence for a non-bile salt dependent fraction in the isolated perfused rat liver. *Gastroenterology.* 59: 853.
- Erlinger, S., D. Dhumeaux, P. Berthelot, and M. Dumont. 1970. Effect of inhibitors of sodium transport on bile formation in the rabbit. *Amer. J. Physiol.* 219: 416.
- Small, D. M. 1970. The formation of gallstones. *Advan. Intern. Med.* 16: 243.
- Mysels, K. J. 1969. Contribution of micelles to the transport of a water-insoluble substance through a membrane. *Advan. Chem. Ser. No.* 86, 24.
- O'Maille, E. R. L., T. G. Richards, and A. H. Short. 1965. Acute taurine depletion and maximal rates of hepatic conjugation and secretion of cholic acid in the dog. *J. Physiol. (London).* 180: 67.
- Tamesue, N., and K. Juniper, Jr. 1967. Concentrations of bile salts at the critical micellar concentration of human gallbladder bile. *Gastroenterology.* 52: 473.
- Nilsson, S., and T. Scherstén. 1970. Influence of bile acids on the synthesis of biliary phospholipids in man. *Eur. J. Clin. Invest.* 1: 109.
- Balint, J. A., D. A. Beeler, E. C. Kyriakides, and D. H. Treble. 1971. The effect of bile salts upon lecithin synthesis. *J. Lab. Clin. Med.* 77: 122.